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Regulation of Tumor Invasion and Metastasis in Protein Kinase C Epsilon-Transformed NIH3T3 Fibroblasts

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Abstract Protein kinase C epsilon is an oncogenic, actin nucleating protein that coordinately regulates changes in cell growth and shape. Cells constitutively expressing PKCε spontaneously acquire a polarized morphology and extend long cellular membrane protrusions. Here we report that the regulatory C1 domain of PKCε contains an actin binding site that is essential for the formation of elongate invadopodial-like structures, increased pericellular metalloproteinase activity, in vitro invasion of a Matrigel barrier, and the invasion and metastasis of tumors grown in vivo by PKCε-transformed NIH3T3 fibroblasts in nude mice. While removing this small actin binding motif caused a dramatic reversion of tumor invasion, the deletion mutant of PKCε remained oncogenic and tumorigenic in this experimental system. We propose that PKCε directly interacts with actin to stimulate polymerization and the extension of membrane protrusions that transformed NIH3T3 cells use in vivo to penetrate and degrade surrounding tissue boundaries. J. Cell. Biochem. 85: 785–797, 2002.

Key words: protein kinase C; actin; membrane protrusion; invadopodia; metalloprotease; mRNA stability; mitogenicity

As transformed cells progressively become more proliferative and highly invasive, a complex molecular machinery is assembled that enables the tumor to actively invade dense structural barriers and spread to healthy tissues [Aguirre Ghiso et al., 1999]. During the acquisition of this invasive and metastatic phenotype, distinct, and spatially segregated, multimeric signaling networks are engaged to constitutively activate mitogenic and proteolytic cascades [Aguirre Ghiso et al., 1999]. Among others, the protein kinase C (PKC) family of proteins has been shown to be positively associated with the metastatic spread and invasiveness of human prostate cancer cells [O'Brian, 1998], astroglial brain tumors [Sharif and Sharif, 1999], urinary bladder carcinomas [Busso et al., 1994], gastric cancer cells [Perletti et al., 1998], thyroid carcinomas [Knauf et al., 1999], mammary adenocarcinoma cells [Lavie et al., 1998; Kiley et al., 1999], and murine melanoma cells [LaPorta and Comolli, 1997]. While members of the PKC family play heterologous roles in regulating gene expression, cell growth, proliferation, and apoptosis, PKCε remains the only isoenzyme with established oncogenic activity. Indeed, PKCε has been shown to act as a dominant oncogene in rodent fibroblasts [Cacace et al., 1993; Mishak et al., 1993; Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998; Wang et al., 1998], colonic epithelial cells [Perletti et al., 1998], and hepatocytes [Perletti et al., 1996]. PKCε was the first member of the novel (classified AGC-IIB) subfamily of lipid-regulated protein serine/threonine kinases to be characterized, and it is presently understood that PKCε may participate in the regulation of diverse cellular

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functions, including the modulation of gene expression [Soh et al., 1999], neoplastic transformation [Cacace et al., 1993; Mishak et al., 1993], mitogenicity [Cai et al., 1997; Ueffing et al., 1997], cell adhesion [Miranti et al., 1999], and motility [Winder et al., 1998].

To date, gene transfer studies have most carefully detailed the cellular consequences of PKCε hyperactivity in preneoplastic rodent fibroblasts. This substantial body of work demonstrates that PKCε overproduction causes malignant transformation and all characteristically associated changes in structural morphology, serum- and anchorage-dependence of cell cycle progression, and the ability to form tumors in experimental animals [Cacace et al., 1993; Mishak et al., 1993]. While the molecular basis for this disordered growth control remains controversial; there is now evidence that PKCε may be capable of directly activating the classic mitogenic signaling pathway involving Ras and Raf-1 [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. More recently, transforming growth factors β (TGF-β) have been proposed as alternative candidates for the downstream effector of PKCε [Cai et al., 1997; Ueffing et al., 1997]. PKCε transformed fibroblasts secrete increased amounts of biologically active TGF-β2, TGF-β3, and an uncharacterized epidermal growth factor-like mitogen, implying that growth autocrine loops may account for the oncogenic activity of PKCε.

Beyond stimulating autocrine and anchorage-independent growth, excessive or inappropriate PKCε activity may contribute to tumor progression by regulating morphogenic processes that are relevant for the invasive and/or metastatic behavior of malignant tumor cells. In previous studies, we investigated the potential involvement of PKCε in cytoskeletal remodeling and reported that this isoenzyme of PKC contains an actin binding motif that positions it within a cytoskeletal matrix where many PKC substrates are localized [Prekeris et al., 1996; Prekeris et al., 1998; Hernandez et al., 2001]. While phorbol esters facilitated the in vitro interactions between recombinant PKCε and purified α-actin [Prekeris et al., 1998], these PKC agonists are not required to observe the morphogenic effects of PKCε overexpression in living cells [Hernandez et al., 2001]. In the present investigation, we have confirmed that PKCε overexpression induces the transformation of preneoplastic NIH3T3 fibroblasts and have extended this work by identifying a specific protein-protein interaction that is required for these transformed cells to penetrate and degrade tissue boundaries in vivo. Of central importance is the observation that the transformation induced by PKCε was contingent on productive interactions between this kinase and the actin-based cytoskeletal network. The biological significance of this protein-protein interaction was confirmed by the demonstration that an internal deletion of the PKCε actin-binding motif completely abrogated the metastatic spread of cancer cells in athymic (*nu/nu*) nude mice, without significantly altering the growth of primary tumors. Our results demonstrate that PKCε relies on this signal peptide to regulate pleiotropic and interdependent cellular processes that are each capable of contributing to the metastatic potential of cancer cells.

MATERIALS AND METHODS

All reagents used in this study were of the highest grade available and purchased from the Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. All media, streptomycin, G418, bovine calf serum, and fetal calf serum were purchased from Invitrogen (Rockville, MD). Athymic (*nu/nu*) male mice were from Charles River Laboratories, Inc. (Wilmington, MA). 3,3′-Dioctadecyloxacarbocyanine perchlorate (Di O) was from Molecular Probes (Eugene, OR). Antisera raised against PKCε (clone C-15) and matrix metalloproteinase-9 (clone M-17) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [6-^{3}H]Thymidine (25 Ci/mmol) was from Amersham Pharmacia Biotech (Piscataway, NJ). NIH3T3 fibroblasts transformed with a constitutively active Harvey-*ras* oncogene was a gift from Dr. C. Kent (University of Michigan; Ann Arbor, MI) and NIH3T3 cells transformed with the v-*src* oncogene were donated by Dr. J. McCubrey (Brody School of Medicine at East Carolina University). The pLXSN retroviral vector was originally obtained from Dr. A.D. Miller (University of Washington; Seattle, WA).

Single-Cell Cloning

NIH3T3 mouse embryo fibroblasts (ATCC No. CRL-1658) were infected with pLXSN recombinant retrovirus (L) or pLXSN harboring genes for either wild-type PKCε (Lε) or a PKCε mutant

containing an internal deletion from codons 1013 to 1039 of the mouse PKCε gene (corresponding to an actin binding motif; accession number AF028009) that has been designated LΔε. The pLXSN vector contains an SV40 driven *neo* gene, ampicillin resistance marker, and 5′ viral LTR promoter controlling stable overexpression of PKCε. Polyclonal populations of infected cells were selected using the aminoglycoside antibiotic G418 (500 μg/ml) and subcloned by limiting dilution [Prekeris et al., 1998]. The NIH3T3 fibroblasts and all clonal derivatives were routinely grown in Dulbecco's modified Eagle medium (DMEM) with glucose (4.5 g/L), sodium bicarbonate (3.7 g/L), the antibiotics penicillin (100 U/ml) and streptomycin (100 μg/ml), and 10% bovine calf serum (CS).

Assays of Growth in Culture

Cells were seeded at a density of 2×10^4 cells per 35 mm plate in 3 ml of DMEM plus 10% CS and maintained with media changes every 2 days. The average doubling time was determined by counting the total number of cells per plate, in triplicate, at 24 h intervals. Viable cells were identified by trypan blue exclusion and counted using a hemacytometer. The appearance of transformed foci was scored using an inverted phase-contrast microscope at 4 × magnification. Estimates of cell growth rates were based on measurements collected after an initial lag period, and during the log phase, of cell proliferation. Saturation density was measured after 4 days of postconfluent growth with daily medium changes. To assay serum-independent cell proliferation, NIH3T3 cells were preconditioned for 2 days in DMEM containing 0.5% CS to ensure correct depletion of serum; the cultures were then replated, in triplicate, on day 0 in the same medium at 2×10^4 cells per plate. Assays of anchorage-independent growth were performed by suspending 2×10^4 cells in 2 ml 0.33% Bacto-agar in DMEM containing 10% fetal calf serum (FCS) and overlaid onto a 7 ml layer of 0.5% agar on 60 mm culture dishes. These dishes were placed into a humidified 100 mm plate and the agar was supplemented with 0.5 ml DMEM plus 10% FCS every 4 days. After 14 days, colonies were stained and counted.

Tumorigenicity Assays

Nude male mice were inoculated subcutaneously, into the dorsal flanks left and right of the midline, with 1×10^6 cells suspended in 200 μl phosphate-buffered saline (PBS) per site and routinely inspected for tumor growth and morbidity for up to 90 days. The cell cultures used in these studies were free of *Mycoplasma* contamination. When evident, solid tumors were measured with a caliper on the days indicated. The animals were sacrificed when tumors had grown to a considerable size, but before the tumors caused visible distress to their hosts. Histological examinations were performed, in a blinded manner, by a professional histopathologist (John F. Bradfield, DVM, East Carolina University, Greenville, NC) using standard methods. Four-micron thick sections of formalin-fixed tissue were cut and stained with hematoxylin and eosin for examination by light microscopy.

Establishment of Explant Cell Lines

Tumor-bearing animals were sacrificed, localized primary and metastatic tumors were excised using sterile technique, finely minced to form explants, and plated onto 100 mm culture dishes in DMEM containing 10% CS. After a 4 h incubation at 37°C, the explanted tissue was removed by aspiration and adherent cells were washed twice with PBS and propagated in fresh growth media for in vitro passage and characterization. Metastases collected for this purpose were found to be growing as localized hepatic tumors that were well separated from the primary mass.

Mitogenic Activity

Mitogenicity was assayed according to the method of Cacace et al. [1998]. Conditioned medium (CM) was prepared by growing cells to a confluency of ~ 70% in 6-well plates, starving cells for 24 h in DMEM plus 0.5% CS, and then conditioning 1 ml of the same media for 36 h. Aliquots (100 μl) of the CM from donor cells were then transferred to 96-well plates containing confluent cultures of untransformed NIH3T3 cells that had been starved for 24 h, in DMEM plus 0.5% CS, and washed two times with PBS. After 18 h of stimulation with CM, cells were incubated with [^{3}H]thymidine (2.5 μCi/ml) for 3 h, washed, treated with 10% ice-cold trichloroacetic acid (TCA), and DNA precipitates were solubilized with 50 μl of formic acid [Cacace et al., 1998]. [^{3}H]Thymidine incorporation was measured using scintillation counting.

Protrusive Activity of Cells Grown in a Matrigel Matrix

Cells were stained with 5 μM Di O in a suspension of 1×10^5 cells/ml DMEM for 30 min at 37°C. After washing with PBS, equal volumes of the stained cells and Matrigel were gently mixed and 1 ml aliquots of this cell suspension were seeded into pre-cooled 2 well chamber slides (LAB-TEK, Naperville, IL). Cells embedded in this Matrigel matrix were incubated overnight at 37°C and visualized using a Nikon Microphot-FXA fluorescence microscope at 40 × magnification.

Cell Invasion Assays

Assays of in vitro invasion were performed using 24-well Matrigel-coated polycarbonate chambers (Transwell™ 8 μm pore size filters). Cell suspensions (5×10^4 cells in 200 μl) were seeded into the upper chambers of individual Transwell inserts and a chemoattractant (DMEM plus 10% CS) was added to the lower chamber. Cells were allowed to invade for 22 h at 37°C before fixing and staining with Diff-Quick (Baxter, McGraw Park, IL). Cells that had migrated to the under surface of the filter were counted under a 100 × microscope objective.

Zymography Assays

The collagenolytic activities of secreted MMPs were assayed by acrylamide gel zymography. Subconfluent flasks of the specified cell line were washed with PBS and then incubated at 37°C in DMEM plus 0.5% CS for 30 h. The CM was collected and centrifuged at 13,500*g* for 10 min to sediment cell debris and soluble proteins were then precipitated from the supernatant with an equal volume of 100% EtOH at − 20°C for 3 h. Precipitates were solubilized in equal volumes of nonreducing sample buffer (2% SDS, 10% glycerol in 62.5 mM Tris, pH 6.8), and samples were loaded onto a 10% SDS–PAGE gel that had been impregnated with 1 mg/ml gelatin and run at 100 V for 2 h. Caseinolytic activity was assayed by casein zymography according to the method of Heussen and Dowdle [1980]. The SDS was removed, using two 30 min incubations in 2.5% Triton X-100, and incubated for 24–48 h at 37°C in developing buffer (5 mM $CaCl_2$, 1 mM $ZnCl_2$, 50 mM Tris, pH 7.5). Gels were then stained with Coomassie brilliant blue, washed to remove excess dye, dried, and scanned using the Adobe Photoshop™ 5.5 computer program (San Jose, CA).

Assays of mRNA Stability

Stability of the mRNAs for MMP-2 and MMP-9 was measured using the transcriptional block and chase method of Ziegler et al. [1990]. Briefly, subconfluent cultures were exposed to actinomycin D (20 μg/ml) at time 0, to block RNA synthesis, and poly(A) RNA was isolated at the times specified using the Oligotex™ protocol (Qiagen Inc., Valencia, CA). Oligonucleotide probes used for hybridization to the MMP-2 and MMP-9 mRNAs were identical to those employed previously by Greene et al. [1997] and were labeled using [γ-^{32}P]ATP and hybridized according to the KinaseMax™ and Multi-NPA™ protocols, respectively (Ambion, Inc., Austin, TX). Densitometric analysis was performed using the BioRad Gel Doc 2000 Gel Documentation system and Quantity One 4.1.1 software (Hercules, CA).

Immunoblotting

Western blot analyses were performed as described [Prekeris et al., 1998].

Data Analysis

All of the assays performed in this study were conducted at least three times and found to be reproducible. Only representative data are presented and numeric data represent the mean and standard deviation of duplicate or triplicate determinations from at least three independent experiments, as described in the figure legends. Treatment effects were evaluated using a two-sided Student's *t*-test and values of $P < 0.05$ were taken as a significant difference between means.

RESULTS

PKCε Interacts With Actin to Promote Tumor Progression to a Metastatic Stage

NIH3T3 fibroblasts were transformed by constitutively overexpressing the full length PKCε cDNA. The oncogenic potential of PKCε was abundantly clear in our in vitro assays of cellular transformation. Cells that overexpressed PKCε (Lε) were refractile, extended elongate cellular protrusions, formed small dense foci in confluent cultures, and displayed a significantly increased saturation density and

TABLE I. Growth Properties of NIH3T3 Cell Lines Overproducing PKCε or a Deletion Mutant Lacking an Actin-Binding Motif (ΔPKCε)

Cell line	Foci formation	Maximum cell density (cells/cm$^2 \times 10^{-4}$)	Growth in agar	Doubling time (h)	Tumor formation in nude mice	Metastases in nude mice
3T3	–	7.1 ± 1.2	–	16.6 ± 0.5	ND	ND
L	–	9.5 ± 0.9	–	16.3 ± 1.0	0/4	0/4
Lε-3	+	ND	+	ND	8/8	8/8
Lε-5	+	9.8 ± 0.3	ND	16.0 ± 0.3	4/4	2/4
Lε-7	+	15.9 ± 0.3^a	+	13.6 ± 0.5^a	14/16	13/16
LΔε-2	+	ND	ND	ND	4/4	0/4
LΔε-5	+	12.5 ± 0.2^a	++	23.7 ± 1.9^a	ND	ND
LΔε-9	+	19.9 ± 0.7^a	++	21.3 ± 2.5^a	16/16	0/16

ND, not determined.
[a]$P < 0.05$ versus 3T3 control cell line.

anchorage-independent growth in soft agar (Table I). These observations were made in the absence of phorbol esters and were consistent with that previously reported [Mishak et al., 1993; Hernandez et al., 2001]. A pLXSN vector control cell line (L) was indistinguishable from the parental cell line in each of these assays (Table I). To identify determinants of PKCε induced transformation, we have examined the role of specific signal peptides that are unique to this member of the PKC family. In the present study, we have targeted an actin binding motif that is located within the C1 domain of PKCε [Prekeris et al., 1996; Prekeris et al., 1998] using a PCR-based deletion mutagenesis approach. NIH3T3 cell lines overexpressing this deletion mutant (called LΔε) resembled the Lε cell lines in certain respects; these cells were also refractile, formed foci, had an increased saturation density, and grew in soft agar (Table I). In fact, the LΔε cell lines consistently formed larger colonies in soft agar than those formed by the Lε cells (Table I).

The in vivo tumorigenicity that PKCε imparted to NIH3T3 cells did not prove to be dependent on the localization of PKCε within the cytoskeletal matrix, as LΔε variants remained > 90% tumorigenic in nude mice (Table I and Fig. 1A). Remarkably, however, this mutation of PKCε conferred an attenuating phenotype that only became evident during advanced stages of tumor progression. Nude mice inoculated with variants of the Lε cell lines (Lε clones 3 and 7) formed tumors within 5 weeks (Fig. 1A), and histopathologic evaluation of these tumor-bearing mice provided clear evidence of active invasion by the tumor cells into the surrounding tissue (Fig. 2A). Although occasional central areas of necrosis were found in some of the Lε tumors, there were abundant vascular channels scattered throughout all sections (Fig. 2C). Evidence of direct hematogenous metastasis to the liver was also apparent, as nests of tumor cells were found in the centrilobular and periportal areas (Fig. 2E). Indeed, metastases were found in several locations of the mesentery and, within 6 weeks, were present in more than 80% of the mice injected with Lε cells (Table I). In contrast, LΔε cells formed paralumbar (primary) tumors that grew more slowly than Lε tumors (Fig. 1A) and never progressed to an invasive or metastatic stage (Table I). Histological analyses have failed to detect any evidence of LΔε cell dissemination in the heart, lungs, brain, liver, spleen, kidneys, or gastrointestinal tract. The primary LΔε tumors were character-

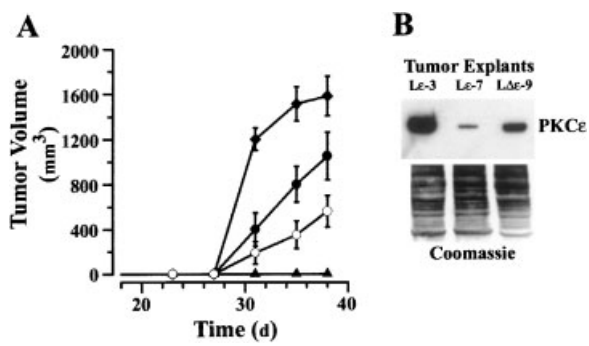

Fig. 1. **A**: Tumorigenic effects of PKCε overexpression in murine NIH3T3 fibroblasts. Each cell line was injected s.c. into male nude mice and the volume of paralumbar tumors was calculated by the formula: length × width × depth × 0.5236. Each data point represents the mean tumor volume in each experimental group containing at least four mice (see Table I). Sublines tested included the NIH3T3 and vector controls (both▲), Lε-3 (◆), Lε-7 (●), and LΔε-9 (○) fibroblasts. **B**, Immunoblot analysis of PKCε expression in Lε-3, Lε-7, and LΔε-9 explants of primary tumors passaged in culture five times prior to the preparation of whole cell lysates, 10 μg total protein loaded per lane. Coomassie-stained gel shown below was a control to confirm equal loading of all lanes.

ized by distinct tumor margins and extensive central necrosis and hemorrhage (Fig. 2B, D). Although the volume of LΔε tumors was initially less than the Lε tumors, this did not account for the apparent absence of LΔε cell metastasis. Respective final volumes of the Lε and LΔε primary tumors ultimately reached 1590 ± 160 mm^3 and 1440 ± 123 mm^3 at the time of sacrifice. Finally, immunoblot analyses of primary tumor explants growing in culture for five passages demonstrated that the level of PKCε and ΔPKCε expression in the host animal did not correlate with the metastatic potential of the transformed NIH3T3 cell lines (Fig. 1B). In these cultured tumor explants, the nonmetastatic LΔε-9 cells expressed an intermediate level of PKCε in comparison to the two metastatic lines examined (Lε-3 and Lε-7). These studies implied that disrupting the interactions between PKCε and the cytoskeleton prevented metastasis but not tumor growth.

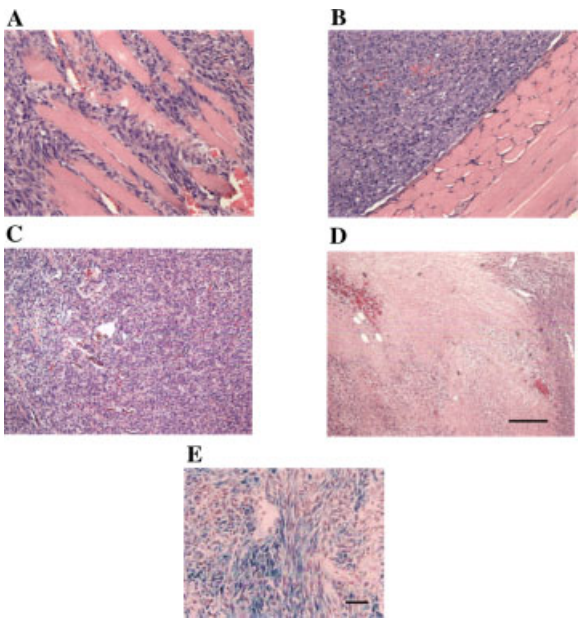

Fig. 2. Histological visualization of tumor cells after microsectioning, staining with hematoxylin and eosin and visualization under 400× magnification. Tumors illustrated were representative of those examined. **A**: Primary tumor established by Lε-7 fibroblasts showing indistinct, expansile borders and extensive tumor infiltration. **B**: Intact boundary between a primary tumor of transformed LΔε-9 fibroblasts (upper left) and abdominal tissue of the host (lower right). **C**: Core region of a Lε-7 tumor with vascular channels and occasional evidence of hemorrhage. **D**: Central necrosis of a LΔε-9 tumor and accumulations of extracellular fibrillar hyalin material interpreted to be collagen. Calibration bar is 20 μm. **E**: Metastatic Lε-7 tumor cells found in a periportal area of the liver 38 d postinoculation. Calibration bar is 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The Lε-7 and LΔε-9 cell lines were selected as representatives in the comparative analyses that follow, unless otherwise indicated. Although their levels of PKCε and ΔPKCε protein expression differed in tumor explants (Fig. 1B), direct comparisons of 16 different Lε and LΔε clones indicated that these two sublines (Lε-7 and LΔε-9) provided the closest approximation in terms of their levels of PKCε protein expression in culture [Hernandez et al., 2001].

PKCε Interacts With Actin to Promote Mitogenicity But Not Autocrine Proliferation

It is presently understood that in rodent fibroblasts, the transforming potential of PKCε is strictly dependent on the production of autocrine growth factors [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. The important finding that mitogens secreted by fibroblasts overexpressing PKCε have a potent stimulatory effect on DNA synthesis supports this model. We have confirmed that CM collected from NIH3T3 cells overexpressing PKCε has a mitogenic effect on the incorporation of [^{3}H]thymidine into parental NIH3T3 cells (Fig. 3A). The PKCε-induced production of autocrine growth factors would also be expected to diminish the requirement of Lε cells for exogenous growth factors or serum [Cacace et al., 1993]. However, growth curves indicated that neither Lε-7 nor Lε-3 cells actively proliferated in the absence of serum, although both were more capable of surviving under these conditions than the LΔε-9, vector control, or parental cell lines (Fig. 3B data not shown). In contrast, the number of viable Ha-*ras* transformed NIH3T3 cells doubled in the absence of serum (Fig. 3B). This was an important result because it indicated that PKCε may be capable of inducing both oncogenic transformation and tumorigenesis without rendering NIH3T3 cells autocrine in growth. Analysis of the LΔε-9 mutants provided additional support for this conclusion. CM collected from LΔε-9 cells had a minor but insignificant effect on the synthesis of DNA by their parental counterparts (Fig. 3A), and the survival of LΔε-9 cells was serum-dependent (Fig. 3B). Therefore, the PKCε-induced transformation of NIH3T3 cells appears to be associated with an increased production of mitogenic growth factors but does not engage the full program required for

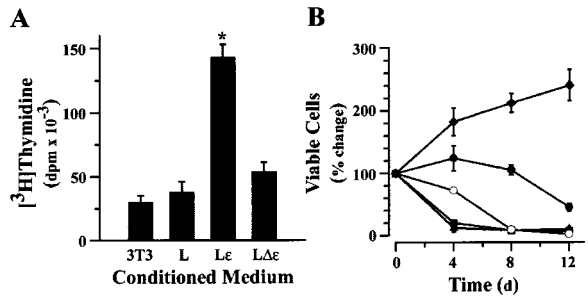

Fig. 3. **A**: [^{3}H]Thymidine incorporation by NIH3T3 cells following an 18 h exposure to medium that had been preconditioned by parental (3T3) or vector (L) controls and the Lε-7 (Lε) or LΔε-9 (LΔε) cell lines. Data are the mean with SD of triplicate determinations from three independent experiments. **B**: Proliferation of NIH3T3 (■), vector controls (▲), and NIH3T3 cells overexpressing Lε-7 (●), LΔε-9 (○), or Ha-Ras (◆) in the presence of 0.5% serum for the time indicated. Cell proliferation was measured by counting the total number of viable cells per plate by trypan blue exclusion. Data are the mean with SD of triplicate determinations from three separate experiments.

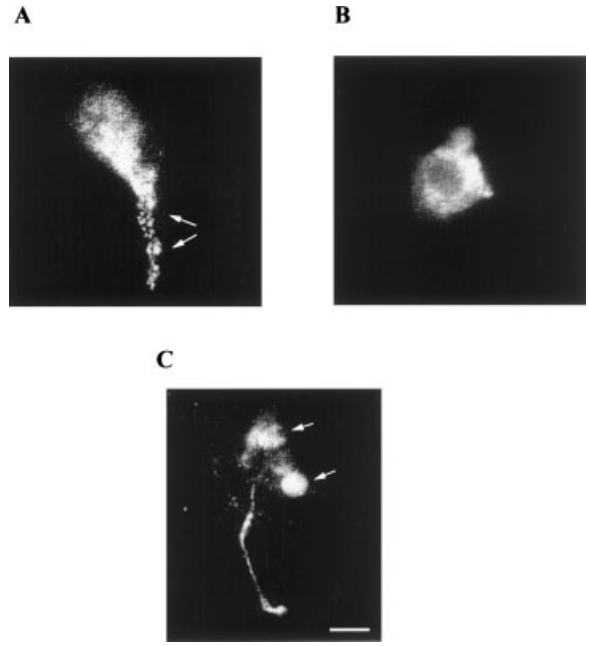

Fig. 4. **A**: Fluorescence images of an invadopodial-like extension (arrows) from the Di O-stained plasma membrane of a Lε-7 fibroblast after suspension for 24 h within a three-dimensional Matrigel matrix. **B**: Di O-stained LΔε-9 cells grown under identical conditions to those used in A. **C**: Digital image of a Di O-stained Lε-7 cell penetrating an 8-μm pore of a Matrigel coated membrane, as viewed from the undersurface. Two presumptive pores (arrows) mark sites of potential cellular invasion and entry into the lower chamber of the Transwell insert. Calibration bar is 10 μm and images are representative of either six (A and B) or two (C) independent experiments.

genuine autocrine growth. Host-derived growth factors may be responsible for supporting the in vivo growth of Lε and LΔε primary tumors.

PKCε Interacts With Actin to Extend Invadopodia and Augment the Invasiveness of NIH3T3 Fibroblasts

Although NIH3T3 and neuroblastoma cells that overexpress PKCε rapidly form elongate cellular processes on a tissue culture dish [Fagerstrom et al., 1996; Prekeris et al., 1996; Zeidman et al., 1999], it remained unclear whether these cells would also form the invadopodial-like extensions that are characteristic of invasive tumor cells [Monsky et al., 1994]. For this reason, we examined the morphology of NIH3T3, L, Lε, and LΔε cells that had been stained with a fluorescent membrane dye, Di O, and cultured overnight in a complex and three-dimensional biological matrix of the Engelbreth-Holm sarcoma-derived Matrigel [Albini et al., 1987]. As illustrated in Figure 4A, Lε-7 cells formed long thin protrusions into the Matrigel that closely resembled invadopodia [Monsky et al., 1994], while the LΔε-9 clone had maintained a completely rounded morphology within this three-dimensional matrix (Fig. 4B). Under these conditions, parental and vector controls also remained rounded and did not form membrane extensions. Digital images of the invasive Lε-7 cells captured several instances in which membrane extensions at the leading edge of the cell were actually seen to be penetrating through the 8 μm pores of a Matrigel membrane while traversing into the lower chamber of a Transwell apparatus. Figure 4C shows an example of this invasive behavior, where an elongate invadopodial extension can be seen protruding through a pore of the Matrigel membrane. This visual evidence provided convincing proof that PKCε-transformed NIH3T3 cells could employ their invadopodial-like processes to traverse an artificial extracellular matrix (ECM).

A variety of cytoskeleton-dependent processes become involved in regulating the attachment, migration, and invasion of tumor cells during the progressive stages of metastasis. However, our results indicated that changes in cell adhesion and motility could not account for the invasive phenotype of PKCε-transformed NIH3T3 cells. Lε cells were more adherent on a fibronectin matrix than their parental cells, apparently due to an upregulation of integrin β1 avidity, and less motile in chemotaxis, chemokinesis, and cell scattering assays [Mayhew

et al., unpublished communication]. Accordingly, we proceeded to investigate the effect of PKCε overexpression on the production of activated proteases. A variety of ECM-degrading proteases have been associated with invadopodia [Monsky et al., 1994; Chen, 1996]. To obtain a quantitative assessment of the in vitro invasiveness of PKCε-transformed NIH3T3 cells, we performed invasion assays with Matrigel as the matrix barrier. Using this approach, it was demonstrated that the invasiveness of Lε-7 cells was four-fold greater than the corresponding vector controls (Fig. 5A). Moreover, this striking increase in invasive activity was completely absent in the LΔε-9 deletion mutant (Fig. 5A).

The oncogenes v-*src* and Ha-*ras* coordinately upregulate the expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR) in NIH3T3 cells while downregulating the production of caveolin-1, an integral membrane protein that interacts with uPAR to assemble kinase-rich domains within focal adhesions [Koleske et al., 1995; Aguirre Ghiso et al., 1999; Wei et al., 1999]. An immunoblot analysis of cell lysates prepared from NIH3T3 cells overexpressing v-*src*, Ha-*ras*, PKCε or ΔPKCε revealed that caveolin-1 expression was only decreased in the invasive v-*src*, Ha-*ras*, and Lε sublines (Fig. 5B). To investigate the role of uPA or the MMPs in ECM degradation, we established whether the vector controls and PKCε-transformed NIH3T3 cells differentially secreted these zymogens. Samples of CM from each cell line were assayed for caseinolytic and collagenolytic activity. Figure 5C illustrates that uPA activity did not accumulate in the medium of NIH3T3 cells overexpressing wild-type PKCε, whereas caseinolytic activity was consistently detected in the medium conditioned by vector controls and LΔε-9 cells. Therefore, the invasive behavior of PKCε-transformed NIH3T3 cells did not appear to depend on the upregulation of caseinolytic activity. We next examined the effect of this oncogene on the production of the type IV collagenases/gelatinases MMP-2 and MMP-9. The mouse homologue of proMMP-9 (gelatinase B) is an inactive zymogen of 105 kDa [Tanaka et al., 1993]. Using an antibody that only recognizes this latent form of MMP-9, it was determined that the ectopic overexpression of wild-type PKCε, but not ΔPKCε, increased the secretion of proMMP-9 without altering the cellular levels of this protein (Fig. 5D). This result reflects the fact that fibroblasts constitutively secrete proMMP-9. At the same time, however, the 105 kDa form of this collagenase was not the major species detected in zymograms of the CM from either the control or PKCε-transformed NIH3T3 cell lines. Rather, we consistently detected the presence of a secreted 96–98 kDa fragment of murine MMP-9 activity that was not upregulated in PKCε overexpressing clones relative to the control cell lines under basal growth conditions (Fig. 5E). It is also important to note that MMP-9 activity in CM from LΔε-9 cell cultures was significantly decreased and was not consistently detected under these conditions (Fig. 5E, lane 4). Immunoblot analyses were also performed using antibodies raised against MMP-2; however, we were unable to detect immunospecific bands for this proteolytic enzyme. Gelatin zymographies, on the other hand, clearly showed that CM from Lε-7 and LΔε-9 clones contained increased levels of collagenolytic activity corresponding to 70–76 kDa proteins, possibly including MMP-2 (Fig. 5E). The collagenolytic activities of both the 70–76 kDa and 96–98 kDa proteins were inhibited by the addition of either EDTA or the synthetic MMP inhibitor phenanthroline to the CM (data not shown). Moreover, ultracentrifugation of the CM did not lower the levels of soluble MMP-2 (70–76 kDa) or MMP-9 (96–98 kDa) activity (data not shown); implying that neither MMP was primarily associated with membrane vesicles [Ginestra et al., 1998]. These data indicate that the production of endogenous MMPs but not uPA was upregulated in PKCε-transformed NIH3T3 fibroblasts. In comparison to the nonmetastatic LΔε-9 cells, Lε-7 cells produced increased levels of both MMP-2 and MMP-9 activity when cultured under identical conditions (Fig. 5E).

Because tumor cell interactions with the surrounding stroma play such a central role in regulating the in vivo expression of type IV collagenases, CM from tumor explants were also assayed for collagenolytic activity. Explants from primary (paralumbar; Lε-7 and LΔε-9) and secondary (hepatic metastasis; Lε-7) tumors were cultured in serum-free medium for 30 h and samples of their CM were analyzed using gelatin zymography. In comparison to the CM from a secondary Lε-7 tumor explant, medium conditioned by the primary tumor explant had accumulated higher levels of collagenolytic

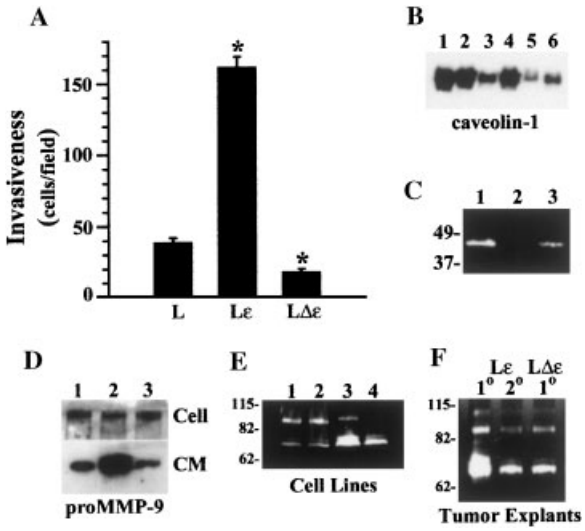

Fig. 5. **A**: In vitro invasion of PKCε-transformed NIH3T3 fibroblasts. Cell suspensions (5×10^4 cells in 200 μl) of vector controls (L) or cells overexpressing either PKCε (Lε) or an actin binding null mutant (LΔε) were placed into the upper wells of individual Transwell inserts containing 8-μm pore size polycarbonate membranes precoated with Matrigel (0.78 mg/ml). Cells were allowed to invade for 22 h at 37°C followed by the fixation and staining of cells. Cells that passed through the membrane were counted at 100× magnification. Data are the mean with SD of triplicate determinations in four separate experiments. **B**: Western blot analysis of caveolin-1 expression in parental and vector control cells (**lanes 1 and 2**, respectively) and in NIH3T3 cells that have been experimentally transformed by a known oncogene; i.e., PKCε, ΔPKCε, Ha-*ras*, or v-*src* (**lanes 3–6**, respectively). Samples containing an equal amount of protein (50 μg) were loaded into each lane. **C**: Zymographic analysis of caseinolytic activity in CM collected from NIH3T3 cells overexpressing an empty pLXSN vector, PKCε, or ΔPKCε (**lanes 1–3**, respectively). Results are representative of six individual experiments. **D**: Production and secretion of proMMP-9 in NIH3T3 clones, loaded in the order specified in B. Samples of cell lysate (Cell, 25 μg protein) and conditioned medium (CM, 15 μg protein) were loaded into each lane and results are representative of at least two independent experiments. **E**: Gelatin zymography of collagenolytic activity in CM collected from NIH3T3, vector control, Lε-7, and LΔε-9 cells (**lanes 1–4**, respectively). Results are representative of ten experiments. **F**: Gelatin zymography of CM collected from the explants of primary (1°) tumors grown in nude mice inoculated with either Lε-7 cells (**lane 1**) or LΔε-9 cells (**lane 3**) and a metastatic (2°) tumor established in the former animal (**lane 2**). Explants were prepared from four individual animals and this is one representative experiment.

activity (Fig. 5F, lane 1 vs. 2). Both of these tumors were originally taken from the same animal. The nonmetastatic LΔε-9 tumor explant appeared to produce as much MMP-2 and MMP-9 as the secondary Lε-7 tumor (Fig. 5F). These results indicated that the level of collagenolytic activity produced by PKCε-transformed NIH3T3 cells may be influenced by local factors within the surrounding environment.

PKCε Overproduction Selectively Augments MMP-9 mRNA Stability

Several members of the type IV collagenase/gelatinase family (MMP-1, -3, -9, -10, and -11) have been shown to be responsive to PKC activation [Johnson et al., 1999; Sehgal and Thompson, 1999]. In the case of MMP-9, increases in PKC activity have been related to increased levels of total MMP-9 RNA [Crowe and Brown, 1999] that may result from direct effects on gene transcription or the regulation of nontranscriptional pathways [Johnson et al., 1999; Sehgal and Thompson, 1999]. Our interest in the effects of PKCε overexpression on MMP-9 mRNA stability was less than intuitive and only emerged following an extensive analysis of alternative possibilities that are most appropriately summarized at this time. First, we examined the possibility that PKCε might indirectly upregulate the activity of MMPs by stimulating the plasminogen activator systems within the proteolytic cascade [Aguirre Ghiso et al., 1999]. However, we found no evidence of uPA activity in media conditioned by Lε cultures (Fig. 5C). Second, we considered the possibility that PKCε could upregulate the expression of MMP-2 and/or MMP-9 transcripts. In NIH3T3 cells, PKCε signaling transactivates multiple pathways that converge on the serum response element (SRE) in the c-*fos* promoter [Soh et al., 1999], a member of the AP-1 family that has been shown to regulate MMP-9 promoter activity [Crowe and Brown, 1999]. Therefore, we performed a series of transient-transfection assays with NIH3T3, Lε, and LΔε cells using SRE, AP-1, and NF-κB driven luciferase reporter plasmids under various growth conditions. Identical results were obtained using these cells lines (data not shown). Third, we found no evidence of uPA or MMP activity associated with membrane vesicles isolated from the CM of Lε cultures, suggesting that vesicle shedding [Ginestra et al., 1998] did not play a major role in the in vitro invasiveness of these cells. Fourth, biochemical assays revealed that the level of MMP-2, but not MMP-9, pericellular activity was substantially increased in the CM of both invasive Lε-7 and noninvasive LΔε-9 cultures. More importantly, MMP-2 activity appeared to remain elevated in the primary tumors, where MMP-9 activity

could also be detected, and in either case the level of collagenolytic activity was always highest in explants prepared from invasive Lε-7 tumors (Fig. 5F). Most recently, we have tested the hypothesis that excessive PKCε activity might differentially influence the half-life of MMP-2 and MMP-9 transcripts in the invasive Lε-7 cell line. Cells were exposed to actinomycin D (a transcriptional inhibitor) for different times and the levels of the MMP-2 and MMP-9 poly(A)$^+$ mRNA were determined.

As illustrated in Figure 6, Northern blots revealed that the MMP-2 mRNA was significantly more stable than the MMP-9 mRNA in NIH3T3 fibroblasts, with half-lives corresponding to about 24 h and 5 h, respectively (Fig. 6B). Interestingly, while MMP-2 mRNA stability was not altered by the constitutive expression of PKCε in cultured NIH3T3 fibroblasts, the half-life of MMP-9 mRNA was markedly increased in Lε-7 cells (Fig. 6B). Although the stability of MMP-9 mRNA was not assayed in LΔε cells, it is important to note that these fibroblasts did not accumulate detectable levels of MMP-9 activity in culture (Fig. 5E). Therefore, we have shown that the overexpression of full length PKCε in NIH3T3 fibroblasts selectively stabilizes MMP-9 mRNA and augments the secretion of a latent form of this zymogen (Figs. 5D and 6). To investigate whether the prolonged half-life of MMP-9 mRNA can sustain the production of an active protease in the absence of ongoing transcriptional activity, we examined the effects of actinomycin D on the secretion of MMP-9 by Lε-7 cells. Lε-7 cells were incubated for 20 h in the absence or presence of 20 μg/ml actinomycin D and then CM samples were assayed for collagenolytic activity. Under these conditions, MMP-9 secretion was unimpaired (Fig. 6C). In contrast, collagenolytic activity was not detected in CM collected from Lε-7 cells exposed to either 100 μM cycloheximide (an inhibitor of protein synthesis) or 10 μM brefeldin A (an inhibitor of the Golgi secretory pathway) for 20 h (Fig. 6C). These data indicate that the translational control of MMP-9 expression may have an important influence on the invasiveness of PKCε-transformed NIH3T3 cells.

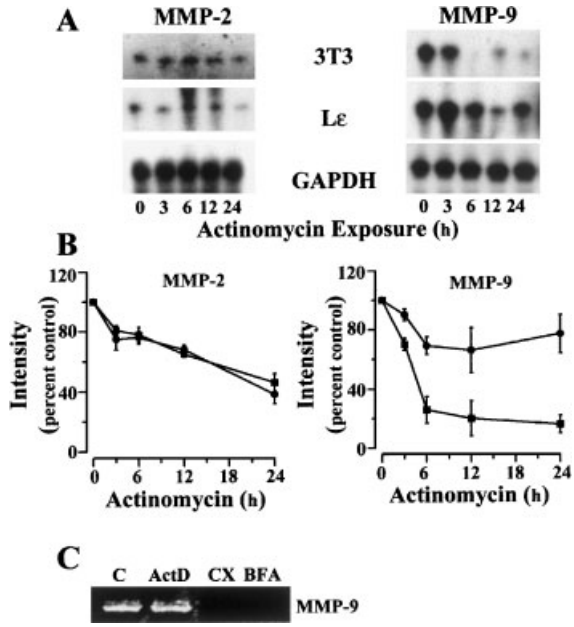

Fig. 6. Northern blot analysis for type IV matrix metalloproteinase transcripts in NIH3T3 cells (■) and transformants overexpressing PKCε (Lε-7;●). **A**: Polyadenylated mRNA (2 μg/lane) was isolated from cultures exposed to actinomycin D (ActD, 20 μg/ml) for the specified time and used to detect the specific mRNA transcripts. A GAPDH probe was used as an internal control. **B**: The BioRad Gel Documentation system was used for densitometric quantitation and the band intensity of untreated cells was arbitrarily defined as 100% control. The data are the mean with SD of three independent experiments. **C**: Gelatin zymography of collagenolytic activity in the CM collected from Lε-7 cells following a 20 h exposure to serum-free medium (C) or the same medium supplemented with 20 μg/ml ActD, 100 μM cycloheximide (CX), or 10 μM brefeldin A (BFA). Results shown are representative of two independent experiments.

DISCUSSION

Elementary features relevant to the program employed by PKCε to constitutively activate cell cycle progression and cellular transformation remain unsolved; however, it now seems clear that this isoenzyme must productively interact with an actin-based cytoskeleton to influence the metastatic progression of transformed NIH3T3 fibroblasts. Previous studies have stressed the importance of autocrine signaling in the in vitro transformation and in vivo tumorigenesis that is induced by excessive PKCε activity [Mishak et al., 1993; Cai et al., 1997; Ueffing et al., 1997]. Yet, in the present study it has been demonstrated that ΔPKCε-transformed NIH3T3 cells remained tumorigenic in vivo despite a pronounced reversion of their mitogenicity and autocrine growth capacity. This is a significant result because it shows that the contribution of autocrine growth loops may be dispensable in the programming of PKCε induced transformation and tumorigenesis.

Moreover, this underlines the fact that there can be important differences between in vitro evidence of transformation and in vivo evidence of tumorigenic and metastatic activity. It is speculated that LΔε tumor cells may derive the factor(s) required for tumor growth, but not intravasation, from the host stroma in the model we have employed. Other findings presented in this, and a second [Hernandez et al., 2001], report advance the notion that PKCε may interact with actin to directly promote polymer assembly and the extension of invadopodial-like processes, while indirectly stimulating the production of MMP-2 and MMP-9 zymogens. As a consequence, PKCε may coordinately regulate the metastatic potential of transformed NIH3T3 cells.

Metastatic cascades are characterized by a multistep process in which cytoskeletal rearrangements play a prominent role. The transition to an invasive phenotype occurs relatively late in tumor progression and is associated with a loosening of cell attachment to the primary mass, aberrant motility, membrane protrusion, and penetration of the surrounding stroma or ECM [Monsky et al., 1994; Chen, 1996; Aguirre Ghiso et al., 1999]. Clear histopathologic evidence of active invasion by the Lε tumor cells into host tissue has now been presented, while tissue margins bordering LΔε tumor cells remained intact. These results show that the internal deletion of a small actin-binding motif was sufficient to dramatically attenuate the highly invasive phenotype of PKCε-transformed NIH3T3 fibroblasts. This report features a fascinating aspect of tumor invasion by demonstrating that the regulatory C1 domain of PKCε participates in the generation of protrusive cell shape changes and activation of a proteolytic cascade. Our results support the hypothesis that PKCε activation and actin polymerization are both critical events driving the expression of MMP-9 by invasive tumor cells [Chintala et al., 1998; Hernandez et al., 2001].

Mechanical pressure must be generated to establish the membrane protrusions observed in fibroblasts [Hernandez et al., 2001 and this report] and neuroblastoma cells [Fagerstrom et al., 1996] overexpressing PKCε. It is now abundantly clear that the prerequisite force is created by the N-terminal regulatory domain of PKCε and that this morphogenetic activity is independent of kinase activity and not diminished by truncation of the entire catalytic domain [Zeidman et al., 1999]. Other mechanistic details of the operational machinery are also becoming apparent. Studies with a cell-free system reveal that binary interactions between PKCε and actin promote the de novo nucleation and elongation of new filaments, either by decreasing the critical concentration of actin and/or the off-rate at pointed ends of pre-existing filaments [Hernandez et al., 2001]. Deletion analysis further indicates that the actin binding motif of the PKCε C1 domain designates an essential interface in these protein–protein interactions. Based on these and other observations [Prekeris et al., 1996, 1998], we have proposed that PKCε may generate protrusive forces in a living cell by nucleating de novo polymerization and stabilizing polymerization intermediates [Hernandez et al., 2001], in a manner that would be reminiscent of the mechanism employed by the Arp 2/3 complex for lamellipod extension [Bailly et al., 1999; Borisy and Svitkina, 2000].

Microscopic images have now been captured in which it was evident that Lε, but not LΔε, cells make use of membrane protrusions at their leading edge to penetrate and degrade Matrigel during the in vitro invasion of a microporous membrane. These polarized structures have been called invadopodial extensions, actin-containing protrusions associated with high local concentrations of ECM-degrading proteases [Monsky et al., 1994; Chen, 1996]. Independent biochemical evidence that the processes formed by Lε cells actually contain localized hot spots of protease activity remains to be established. However, we have previously shown that PKCε co-localizes with actin within these membrane extensions [Prekeris et al., 1998], and the present data indicate that MMP-2 and MMP-9 were functional components of the invasive program induced by constitutive expression of PKCε in NIH3T3 fibroblasts. Intracellular signaling pathways associated with transformation and the biogenesis of tumor-associated proteases have recently been reviewed [Aguirre Ghiso et al., 1999]. Here, we propose that PKCε interacts with actin protomers to coordinately penetrate and degrade the natural tissue boundaries imposed by tumor-bearing athymic mice. The later could be accomplished by regulating any number of molecular interactions within the proteolytic cascade [Aguirre Ghiso et al., 1999]. However, these and other studies provide certain clues as to how the positioning of

PKCε within an actin-based cytoskeletal matrix might promote tumor cell invasion. By upregulating the production of TGF-β, prostatic carcinomas manage to increase the half-life of the mature MMP-2 protein and stability of proMMP-9 mRNA [Johnson et al., 1999]. While the importance of TGF-β in the transformation and tumorigenesis of PKCε-transformed NIH3T3 fibroblasts may be debated, several independent laboratories have now reported that TGF-β is secreted by these transformants and contributes to mitogenicity in this experimental system [Cai et al., 1997; Ueffing et al., 1997; Cacace et al., 1998]. We have presented evidence suggesting that PKCε may not be capable of engaging the full program required for genuine autocrine growth of NIH3T3 fibroblasts. As an alternative hypothesis, we propose that the enhanced production of PKCε by NIH3T3 cells might play an important role in regulating the program required for tumor cell invasion. According to this model, PKCε would prompt actin polymerization and cell shape changes through direct protein-protein interactions [Hernandez et al., 2001] and upregulate the expression of TGF-β and other growth factors by phosphorylating cytoskeletal-associated substrates that are capable of transactivating their cognate promoters in a Raf-1 independent manner [Ueffing et al., 1997; Cacace et al., 1998]. Once activated, these growth factors could advance the invasiveness of PKCε-transformed fibroblasts through the post-transcriptional upregulation of MMP-2 and MMP-9 activity.

The major finding of the present study is that the deletion of a small actin binding motif within the C1 domain of PKCε resulted in a clear-cut reversion of the invasive phenotype established by PKCε-transformed NIH3T3 fibroblasts growing in culture or as tumors in vivo. This result strongly suggests that the subcellular localization of PKCε is an important determinant in the progression of tumor invasion and metastasis in this experimental system. Many important details concerning the complex oncogenic signaling cascade that operates downstream of PKCε remain to be established. It is our working hypothesis that PKCε directly interacts with actin to establish membrane protrusions that PKCε-transformed NIH3T3 cells are capable of using to penetrate and degrade tissue boundaries in a primary tumor.

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