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Protein Kinase C δ Involvement in Mammary Tumor Cell Metastasis[1]

Susan C. Kiley, Kimberly J. Clark, Michelle Goodnough, Danny R. Welch, and Susan Jaken[2]

Adirondack Biomedical Research Institute, Lake Placid, New York 12946 [S. C. K., K. J. C., M. G., S. J.], and Jake Gittlen Cancer Research Institute (C7810), Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033 [D. R. W., S. J.]

ABSTRACT

Metastasis requires cytoskeletal remodeling for migration, adhesion, and extravasation of metastatic cells. Although protein kinase C (PKC) is involved in tumor promotion/progression and cytoskeletal remodeling, its role in metastasis has not been defined. PKCδ levels are increased in highly metastatic 13762NF mammary tumor cells (MTLn3) compared with less metastatic, parental cell lines. To determine whether the increase in endogenous PKCδ is functionally related to their increased metastatic potential, we prepared MTLn3 cells that express the inhibitory regulatory domain fragment of PKCδ (RDδ) under the control of a tetracycline-inducible promoter. RDδ expression attenuated endogenous PKC activity, as demonstrated by decreased phosphorylation of the PKC substrate adducin in migrating cells. Thus, in MT cells, RDδ appears to primarily influence cytoskeleton-dependent processes rather than cell cycle progression. To determine whether RDδ expression influenced metastatic potential *in vivo*, MTLn3/RDδ cells were either grown in the mammary fat pad or injected into the tail vein of syngeneic rats, and effects of doxycycline-induced RDδ expression on pulmonary metastases were studied. Consistent with the *in vitro* data, induction of RDδ significantly reduced the number of lung metastases without affecting growth of the primary tumor. These results suggest that interfering with endogenous PKCδ activity by expressing the inhibitory RDδ fragment inhibits cytoskeleton-regulated processes important for MTLn3 cell metastasis.

INTRODUCTION

Metastasis is a multistep process in which tumor cells lose their contacts with neighboring cells, migrate from the primary tumor into the circulation, attach to capillary walls, escape from the vasculature, and invade and colonize other sites to establish secondary tumors (1). To a large extent, the cellular processes associated with metastasis depend on cytoskeletal remodeling. In fact, several cytoskeletal structural and regulatory proteins, such as E-cadherin, α-catenin, β-catenin, rac, and rho, have been implicated in metastatic progression (reviewed in (Refs. 2–4). Identifying molecular mechanisms that regulate the cytoskeleton-dependent processes associated with metastatic progression is key to developing new therapies to treat and prevent metastatic disease.

PKC[3] is a family of phospholipid-dependent ser/thr kinases, the activity of which was linked to tumor cell promotion and progression because they are the major cellular receptors for tumor promoting phorbol esters (reviewed in Ref. 5). Phorbol esters cause rapid changes in cell morphology, cell-cell interactions, and cell migration, indicating that PKC activation regulates cytoskeletal reorganization (6). PKCs also localize to discrete cytoskeletal structures, indicating that they are positioned to interact with and phosphorylate cytoskeleton-associated proteins (7–12). The notion that PKC regulates cytoskeletal functions is further supported by the observation that many PKC substrates in intact cells are cytoskeleton-associated proteins (13–19). By modifying the phosphorylation state and consequently the functions of these cytoskeleton-associated proteins, PKC activation can rapidly influence the organization of cytoskeletal structures.

Most cells express several conventional and novel PKC isozymes, all of which can be activated by phorbol esters. Although PKCs are clearly implicated in tumor promotion/progression and cytoskeletal remodeling, the specific roles, if any, of the individual isozymes have yet to be determined. One approach to studying the roles of individual PKCs in tumor progression is to identify changes in PKC isozyme expression that correlate with progressive stages of transformation. Functional relevance can then be assessed by increasing or decreasing individual PKC activities by overexpressing wild-type or dominant-negative constructs, respectively. In previous studies, we investigated the potential involvement of individual PKCs in mammary tumor progression using related cell lines with different metastatic potentials that were originally derived from 13762NF rat mammary adenocarcinomas (20, 21). Low and moderately metastatic cell lines were derived from tumors growing in the mammary fat pad, whereas the more rapidly growing and highly metastatic MTLn3 cell line was derived from lung metastases. Comparison of the PKC isozyme levels in three related rat MT cell lines demonstrated that PKCδ levels increased 3-fold in the highly metastatic MTLn3 cells compared with less metastatic cell lines (4). To determine whether this increased PKC activity contributed to metastatic potential, we overexpressed PKCδ in the less metastatic cells and demonstrated that increased PKCδ does not affect anchorage-dependent cell growth but significantly enhances anchorage-independent growth. These studies indicated that PKCδ does not directly regulate cell cycle progression but may be involved in attachment-generated growth signals. In complementary studies, we found that expression of the NH_2-terminal regulatory domain of PKCδ (RDδ) selectively interfered with PKCδ localization and activity at cell peripheries. These studies in less metastatic cells established the efficacy of RDδ as a PKCδ inhibitor and suggested a functional link between PKCδ activity and metastatic progression.

In the present investigation, we tested the hypothesis that excessive or inappropriate activation of PKCδ is functionally significant in metastasis of mammary carcinoma cell lines *in vivo*. To do this, we constructed cell lines from the highly metastatic MTLn3 cells with inducible expression of the inhibitory regulatory domain fragment of PKCδ (RDδ). In culture, induced expression of RDδ inhibited anchorage-independent growth, cell attachment, motility, and invasion but had no effect on anchorage-dependent growth of MTLn3 cells. In animals, inducing RDδ significantly reduced lung metastases but had no effect on growth of MTLn3 primary tumors in the mammary fat pad. Taken together, our results demonstrate that RDδ inhibits metastases by interfering with cytoskeleton-dependent processes that are also relevant for attachment, migration, and anchorage-independent growth of cultured cells.

MATERIALS AND METHODS

Materials. α-MEM, FBS, select agar, collagen type IV, and G418 sulfate were purchased from Life Technologies, Inc. (Grand Island, NY). *Trans*IT-100

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[2] To whom requests for reprints should be addressed, at Department of Pathology, Medical Alumni building, A249, University of Vermont, Burlington, VT 05405. Phone: (802) 656-9496; Fax: (802) 656-8892; E-mail: sjaken@zoo.uvm.edu.

[3] The abbreviations used are: PKC, protein kinase C; FBS, fetal bovine serum; TRITC, tetramethyl rhodamine isothiocyanate; Dox, doxycycline; ECM, extracellular matrix; MT, mammary tumor; HA, hemagglutinin antigen; pSer660, phosphoserine-660; STICK, substrate that interacts with C-kinase; wt, wild type.

was procured from Pan Vera Corp. (Madison, WI). Mouse anti-HA monoclonal (clone 12CA5) was from Boehringer-Mannheim (Indianapolis, IN). Rabbit anti-pSer660 adducin antiserum was raised to a phosphopeptide containing the PKC phosphorylation site in α-, β-, and γ-adducins. The antiserum was affinity purified and shown to be phosphorylation-state selective (5). Horseradish peroxidase-conjugated secondary antibodies and ECL detection reagents were from Amersham Life Science, Inc. (Arlington Heights, IL). Affinity-purified FITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, and TRITC-conjugated goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Female Fischer 344 rats (6–7 weeks of age) were from Harlan Sprague Dawley (Indianapolis, IN). Chemicals were from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.

Cell Culture. MTLn3 (passages 45–47) rat 13762NF mammary adenocarcinoma cells were grown in antibiotic-free α-MEM supplemented with 5% FBS at 37°C in a humidified CO_2 atmosphere, as described previously (4).

Construction of Expression Vectors and Cell Lines. The regulatory domain fragment of PKCδ (RDδ), including a triple repeat of the HA epitope tag sequence (H^3) at the 5′ end, was ligated to the BgIII/*Eco*RV sites of pUHD10–3 (a gift from Dr. Hermann Bujard, Zentrum für Molekulare Biologie, Heidelberg, Germany) to provide inducible expression from the minimal cytomegalovirus promoter-tetracycline operator (22). MTLn3 (passage 45) cells were cotransfected with pUHD10–3 or pUHD10–3/H^3RDδ and pUHD172–1neo, which encodes the reverse tetracycline transactivator and neomycin resistance, at a ratio of 10:1 using *Trans*IT-100. G418-resistant colonies were selected, and Dox-induced HA-RDδ was monitored on immunoblots stained with HA antibody. Dox-inducible clones were selected and subcloned twice by limiting dilution to obtain homogeneous populations. Cells transfected with the empty vector pUHD10–3 were used to control for potential nonspecific effects of Dox treatment. Stable transfectants were maintained in α-MEM medium containing 5% FBS and 175 μg/ml G418 (complete medium).

Sample Preparation and Immunoblot Analysis. For screening RDδ clones, cells in duplicate wells (± Dox treatment) of a 24-well tray were scraped into 150 μl of boiling Laemmli Buffer (23). Cell lysates from 100-mm dish cultures were prepared as described (24). Tissue homogenates were prepared on ice with a handheld tissue homogenizer, using 150–200 mg of frozen tissue/ml homogenization buffer [20 mM Tris-HCl (pH 7.4), containing 0.25 M sucrose, 5 mM EDTA, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 50 mM sodium fluoride, and 1 mM sodium vanadate]. Samples were normalized by protein concentration and solubilized in boiling Laemmli buffer. Cell/tissue proteins were separated on 10% acrylamide gels by SDS-PAGE, electrophoretically transferred to nitrocellulose, and stained with antibodies, as described previously (24). The HA monoclonal (12CA5) antibody was used at 1 μg/ml in TBA [20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and 1% w/v BSA]. Immunoreactive bands were visualized with ECL reagents according to the manufacturer's recommendations.

Immunofluorescence in Whole Cells. To monitor RDδ expression in cells, MTLn3/RDδ cells were grown to 40–50% confluence and then incubated with ±1 μg/ml Dox for 20–24 h. Cells were fixed in 3.7% formaldehyde for 5 min at room temperature and permeabilized in −20°C methanol for 3 min. Immunostaining procedures were as described previously (24). The HA antibody was used at 3.3 μg/ml in PBS containing 1% w/v BSA. After washing, coverslips were incubated with FITC-conjugated goat anti-mouse (1:300) antibodies for 1 h. Slides were observed on a Nikon Optiphot fluorescence microscope equipped with a ×100 objective lens, and photographs were taken with Kodak TMAX 400 black and white film.

Anchorage-dependent Growth. For growth rate studies, 10^4 cells in complete medium were seeded into wells of 6-well trays on day −1 and allowed to attach overnight. The next day (day 0), medium was replaced with complete medium without or with 1 μg/ml Dox to initiate RDδ expression. Triplicate wells were trypsinized and counted daily.

Clonogenic Growth in Soft Agar. Uninduced cells (10^4) in complete medium ± Dox containing 0.33% top agar were plated into triplicate 35-mm wells containing solidified 0.66% agar. Where indicated, Dox (1 μg/ml) was included throughout the 2-week growth assay. At the end of 2 weeks, colonies were stained and counted as described.[4]

Cell Attachment Assay. A microtiter plate assay described by Hansen *et al.* (25) was modified and used to monitor the effect of RDδ transgene expression on MTLn3 cell attachment. Cells (± Dox pretreatment) were detached with trypsin and resuspended to a final concentration of 5×10^5 cells/ml in complete medium. These cells were replated into microtiter plate wells (5×10^4 cells/well) coated with collagen type IV (30 μl of 10 μg of collagen/ml PBS per well). Where indicated, Dox (1 μg/ml) was added back to the Dox-pretreated cells, and cells were incubated at 37°C in a CO_2 incubator for 30 min to 8 h. At each time point, unattached cells were removed, and the vital dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was added (1 mg/ml). Cells were incubated for an additional 2 h at 37°C for complete color development, and attached cell number was estimated according to absorbance at 570 nm as described previously.[4]

Zymography. Vector control and RDδ cell cultures at 80% confluence were rinsed once with PBS and then incubated in the absence or presence of 1 μg/ml Dox in 5 ml of serum-free α-MEM containing 10 mM HEPES (pH 7.4) and 5 μg/ml insulin for 16 h. Cell-free conditioned medium (3 ml) was concentrated to 50 μl in Centricon-30 filtration units (Amicon, Inc., Beverly, MA). Aliquots (25 μl) were mixed with nonreducing Laemmli buffer and electrophoresed through collagen-embedded gels as described (26). After electrophoresis, the collagen/acrylamide gel was washed, incubated at 37°C to activate the proteases, and stained according to described methods to visualize regions of digested collagen (26).

Invasion Assay. Dox effects on invasive capacity were assayed by plating cells (± Dox pretreatment) into the upper well of Costar Transwell units in serum-free medium and monitoring cell migration through ECM-coated filters toward FBS-supplemented medium ± Dox. The upper side of the Transwell membrane (pore size, 8.0 μm) was coated with collagen type IV (0.25 μg of collagen/ml PBS per well). Cells were grown in the absence or presence of 1 μg/ml Dox for 24 h, harvested with trypsin, and resuspended in serum-free α-MEM containing 10 mm HEPES (pH 7.4) and 0.1% w/v BSA. The number of cells/cm^2 seeded to Transwells was doubled (relative to the number of cells used in attachment assays) to compensate for the decreased efficiency of RDδ cell attachment (± Dox). Aliquots (10^5 cells) were plated into the upper well of the collagen-coated Transwell inserts placed into wells containing media ± FBS and ± Dox. Cells were incubated for 40 h at 37°C, medium was aspirated, and cells on the upper side of the membrane were removed with a cotton swab. The lower well and underside of Transwell membrane were washed twice with PBS before cells were fixed with 5% formaldehyde in PBS for 10 min. Cells were stained with crystal violet and counted at ×200. Four fields were counted for each of the triplicate membranes.

***In Vivo* Metastasis Assays.** To determine the effect of RDδ on MTLn3 cell metastasis, parental and variant MTLn3 cells were injected into the mammary fat pad or into the tail vein (20, 26). MTLn3 (passage 45) or MTLn3/RDδ clone 51 (passage 5) cells were cultured in the absence or presence of 1 μg/ml Dox for 24 h. Cells were trypsinized, counted, washed twice, and resuspended in ice-cold sterile PBS. For mammary fat pad injections, animals were anesthetized lightly with Ketaset Rompun and injected s.c. with 10^6 viable cells in 0.5 ml of PBS. Where indicated, Dox (200 μg/ml in 2.5% sucrose) was administered in the drinking water. Tumors were palpitated every 3–4 days after the first week and measured with calipers (mean tumor diameter, square root of tumor length × width, in mm) to quantitate tumor growth. Animals were killed with Euthasol on day 25 after tumor cell injection. Tumors growing at the site of injection were measured and then removed and rinsed in ice-cold PBS. Pieces of tumor tissue were rapidly frozen in liquid nitrogen and subsequently used to prepare tissue homogenates for immunoblot analysis. Lungs were removed, rinsed in ice-cold PBS, and fixed in Bouin's solution for 24 h. Metastatic lesions were counted using a dissecting microscope.

For tail vein injections, 10^5 cells in 0.2 ml PBS were injected into the lateral tail vein. Where indicated, Dox was administered in the water supply as described above. Animals were sacrificed with metofane overdose on day 9 after injection. Lungs were removed, rinsed in cold PBS, and fixed in Bouin's solution to quantitate surface lesions.

[4] S. C. Kiley, K. J. Clark, S. K. Duddy, D. R. Welch, and S. Jaken. Protein kinase C δ promotes mammary tumor cell progression toward a metastatic phenotype, submitted for publication.

Cell Migration and Adducin Phosphorylation. To monitor the effect of RDδ expression on PKC-mediated phosphorylation of adducin during cell migration, cells were grown to 80% confluence in 24-well trays and on coverslips. Where indicated, 1 μg/ml Dox was added to cultures for 24 h before scraping an artificial wound into the cell monolayer. Monolayers were washed once with PBS to remove dislodged cells, and medium was replenished. Dox was added back to cultures that had been pretreated with Dox for 24 h, and aphidicolin (10 μg/ml) was added to half the cultures to inhibit cell growth. At 6, 12, 18, and 24 h after wounding the monolayer, cells in 24-well trays were rinsed with PBS, fixed in 5% PBS-buffered formaldehyde for 10 min, and stained with 0.1% w/v Crystal Violet for 5 min. There was no evidence of aphidicolin toxicity at the 24-h time point. The 24-well plates were individually photographed and printed on 8 × 10-inch paper for enlargement so that the wound diameter could be measured with a ruler.

Coverslips were fixed as described previously at 6, 12, and 24 h after wounding the cell monolayer and incubated with affinity-purified rabbit pSer660-adducin antibody (20 μg/ml) and mouse anti-HA antibody (0.33 μg/ml) for double-label immunostaining. After washing, coverslips were incubated with FITC-conjugated goat anti-rabbit IgG (1:300) and TRITC-conjugated goat anti-mouse IgG (1:5000) antibodies. Coverslips were observed on a Nikon fluorescence microscope equipped with a ×100 objective lens and appropriate filters for fluorescein and rhodamine.

RESULTS

RDδd Expression in MTLn3 Clonal Cell Lines. In previous studies, we found that PKCδ message and protein levels were increased in highly metastatic MTLn3 cells compared with related, less metastatic MT cell lines.[4] To determine whether the increase in PKCδ plays a functional role in promoting MT cell growth and metastasis, we generated clones of MTLn3 cells expressing the inhibitory (dominant-negative) regulatory domain fragment of PKCδ (RDδ). The construct includes the HA epitope-tag sequence and was expressed from a tetracycline-inducible promoter system (22). MTLn3/RDδ clones were selected for G418 resistance and cloned by limiting dilution. The clones used in these studies express minimal levels of HA-RDδ in the absence of Dox and ≥10-fold higher levels in the presence of Dox (Fig. 1). Induction of RDδ was dose dependent, and Dox was not toxic at the concentration used (data not shown; however, note that Dox did not inhibit soft agar growth, invasion, or migration of vector control cells; Tables 1, 2, and 5). Inducible homogeneous RDδ expression was retained by all clones for at least eight passages (through passage 15 for clone 51), and all experiments were performed with passage 4 to 7 cells unless indicated otherwise. "Vector" refers to MTLn3 cells cotransfected with empty expression plasmid and the neomycin-resistance plasmid, which were then selected for G418 resistance. Vector cells were not significantly different from wt MTLn3 cells with respect to cell attachment, collagenase activity, or invasion, indicating that transfection and expression of the tetracycline regulator had no significant effects on the behavior of cultured MTLn3 cells (Table 2 and data not shown).

Effects of RDδd Expression on MTLn3 Cell Growth and Attachment. To determine whether the inhibitory RDδ construct directly influences cell growth, we compared the growth kinetics of vector control and RDδ-expressing MTLn3 cells (Fig. 2). Growth rates and doubling times were similar in the presence or absence of Dox, indicating that neither Dox nor RDδ interfere with cell growth. The number of RDδ cells (−Dox) on day 1 was consistently lower than the number of cells seeded, and Dox potentiated this decrease. This did not occur with vector control cells, which suggests that the RDδ cell lines do not attach to plastic as efficiently as vector control cells. Induced RDδ expression caused a small but significant decrease in saturation density from 9.5×10^5 to 7.1×10^5 cells/cm^2. These results demonstrate that RDδ does not directly influence growth rate but may affect other cell functions, such as substratum attachment.

To directly test the effects of RDδ on attachment, we monitored wt- and RDδ-MTLn3 cell attachment to four different ECM proteins. After a 1-h incubation, >60% of the wt MTLn3 cells attached collagen type IV-coated dishes, whereas only 30−40% attached to laminin- or Matrigel-coated dishes, and <10% attached to fibronectin-coated dishes. Dox slightly increased attachment of wt and vector control MTLn3 cells, but this effect was relatively small (<10%). For vector control cells, 95% of the cells were attached 4 h after plating on collagen, and Dox pretreatment did not influence attachment (Fig. 3, *top*). In contrast, even without Dox pretreatment, <50% of the RDδ21 and <20% of the RDδ51 cells were attached 8 h after plating (Fig. 3). Treatment with Dox further reduced cell attachment to 25 and 7.5% for RDδ21 and RDδ51 clones, respectively. The difference between the vector control, RDδ21 cells, and RDδ51 cells in the absence of Dox treatment may be due to a low level leakiness of the expression system. Dox-induced inhibition of attachment in RDδ but not vector control cultures provides strong evidence that RDδ interferes with molecular events required for cell substratum attachment in MT cells.

Effect of Ectopic RDδ Expression on Clonogenic Growth in Soft Agar. Increased growth of cultured cells in soft agar often correlates with increased metastatic potential *in vivo*. To determine whether dominant-negative RDδ could inhibit growth in soft agar, we assayed anchorage-independent growth of vector control and RDδ

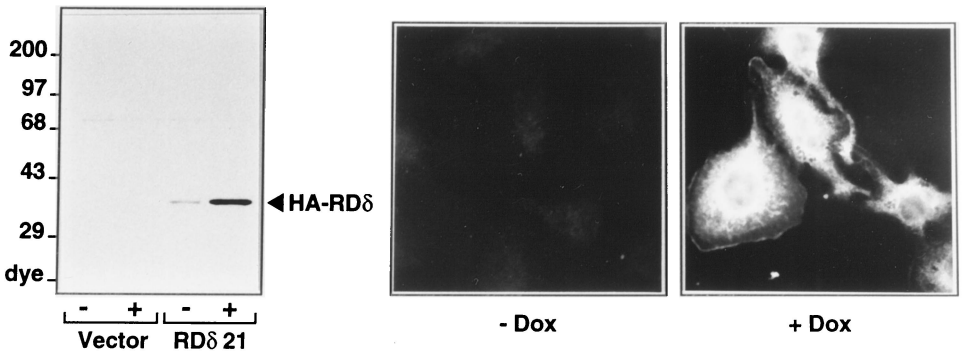

Fig. 1. Expression of the epitope-tagged RDδ construct. *Left*, cell lysates were prepared from duplicate cultures of G418-resistant MTLn3 cell lines grown in the absence (−) or presence **(+)** of 1 μg/ml Dox for 24 h. Cell extracts (50 μg of protein) from vector control and RDδ clone 21 were separated by SDS-PAGE and blotted to nitrocellulose. Immunoreactive bands were identified by probing blots with antibody directed toward the HA epitope tag sequence (*arrowhead*). Left, molecular weight standards (in thousands). Films from blots were scanned on a densitometer and quantitated according to absorbance. *Right*, MTLn3/RDδ 21 cells grown on coverslips were treated ± 1 μg/ml Dox for 24 h. Cells were fixed and stained with anti-HA monoclonal antibody and FITC-conjugated goat anti-mouse IgG, as described in "Materials and Methods." Photographs were taken with a ×100 objective lens. Identical results were obtained in at least three experiments.

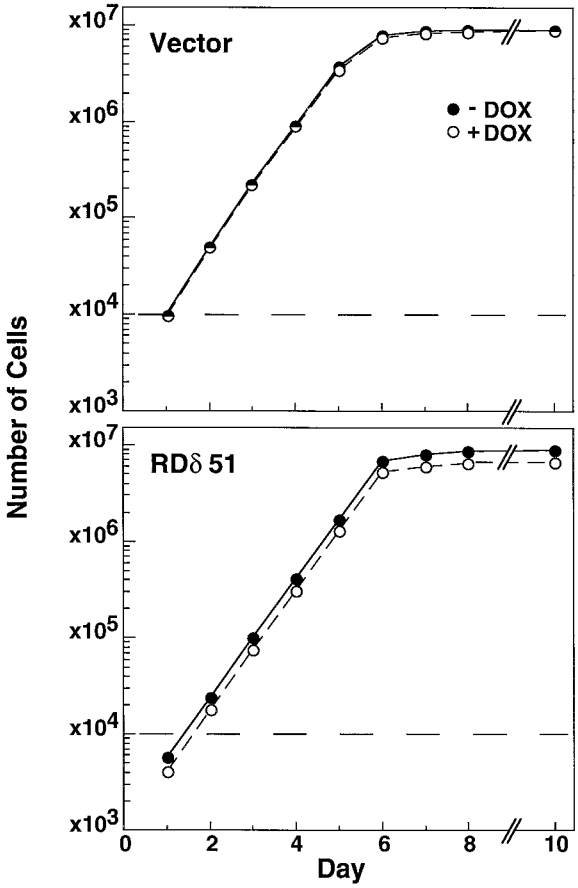

Fig. 2. Effect of RDδ expression on MTLn3 cell growth. Cells (without Dox pretreatment) were seeded at 10^4 cells/well (*dashed horizontal line*) in six-well trays on day −1. After attaching overnight, medium was replaced with medium ± Dox (day 0), and cells were grown in the absence (●) or presence (○) of Dox for the indicated times. Growth rates and saturation densities were compared in cells transfected with the empty vector (*top*) and RDδ (clone 51, *bottom*). Cell numbers were determined in triplicate, and the curves shown are representative for vector control and all three RDδ clones in two independent experiments.

cells in the absence or presence of Dox. Dox did not affect the number of vector control colonies formed in semisolid medium (Table 1). In contrast, Dox-induced RDδ expression reduced the number of colonies to 24–37% of the corresponding control cultures (Table 1). These results support the hypothesis that RDδ expression inhibits processes that correlate with metastatic progression *in vivo*.

Effect of RDδd Expression on Collagenolytic Activity and Cell Invasion. Metastatic cells must degrade ECM barriers to enter and exit from the vasculature and colonize secondary tissues. Invasion is in part mediated through secretion of metalloproteinases that break down ECM. To determine whether RDδ expression influences metalloproteinase levels, we monitored secreted collagenase activity by zymography. The type IV collagenolytic activities detected in vector control and three RDδ cultures were identical with or without Dox treatment (Fig. 4). These results indicate that RDδ expression has no measurable effect on metalloproteinase secretion or activity.

Cell motility is another cytoskeleton-dependent process required for metastasis. We used an *in vitro* assay for cell migration through collagen-coated membranes to evaluate the effect of RDδ expression on MTLn3 cell motility. In this assay, invasion involves attachment to and migration through ECM-coated filters toward FBS-supplemented medium in the lower chamber. Dox treatment profoundly inhibited invasion of RDδ21 and RDδ51 clones, reducing the number of invading cells from 200 to 300 to <2 cells/field (Table 2). Because RDδ expression did not reduce metalloproteinase activity, these results indicate that RDδ interferes with other cellular processes relevant to invasion, such as cell motility.

Effect of RDδd Expression on MT Growth and the Development of Metastatic Lesions. The studies with cultured cells indicate that although RDδ expression does not influence cell growth rates, it does interfere with several correlates of tumor progression and metastasis: anchorage-independent growth, cell attachment, motility, and invasion. However, because metastasis is a complex series of processes, metastatic potential cannot be fully evaluated in cell culture studies; animal studies are needed for complete evaluation of meta-

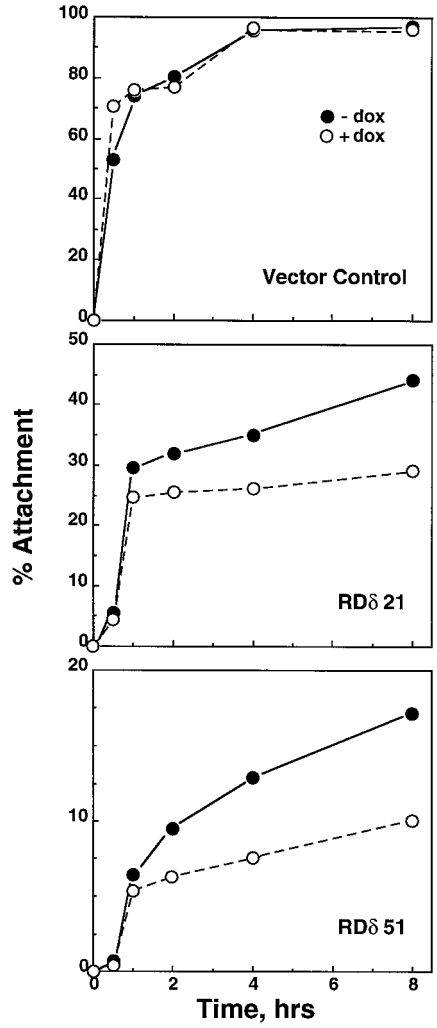

Fig. 3. Effect of RDδ on cell attachment. MTLn3 cell cultures were preincubated in the absence (●) or presence (○) of Dox for 24 h to induce expression of the inhibitory RDδ fragment. Cells were trypsinized, and 5×10^4 cells were plated into microtiter trays precoated with collagen type IV. Attached cells were quantitated as described in "Materials and Methods" and expressed as a percentage of total cells/well. Identical results were obtained in two additional experiments.

Table 1 *Effect of RDδ on MTLn3 cell clonogenic growth in soft agar*

	Colony number, mean ± SD			
Cell line	Vector	RDδ21	RDδ36	RDδ51
−Dox	1127 ± 193[a]	901 ± 46	962 ± 81	667 ± 87
+Dox	1130 ± 198	232 ± 66	360 ± 91	163 ± 39
% control[b]	100	26[c]	37[c]	24[c]

[a] Values shown are numbers of colonies (mean ± SD) for $n = 6$ individual wells from two independent experiments.
[b] Percentage of colonies in Dox-induced cultures relative to control (−Dox) cultures.
[c] $P \leq 0.001$, by Student's t test.

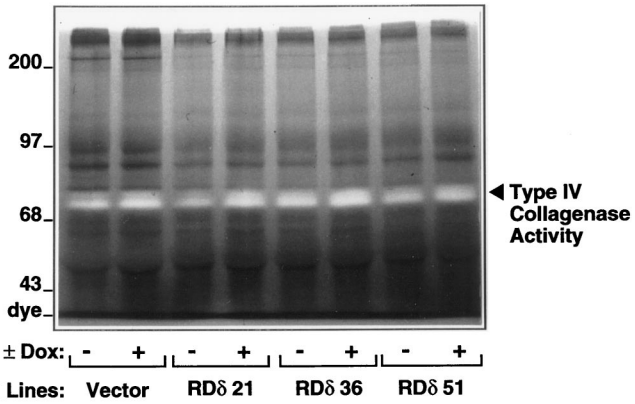

Fig. 4. Effect of RDδ on secreted type IV collagenolytic activity. Vector control and three different RDδ clones were cultured in the absence (−) or presence **(+)** of Dox for 16 h. Samples were prepared from the concentrated conditioned medium collected from each culture and electrophoresed into a collagen type IV-embedded acrylamide gel as described in "Materials and Methods." The gel was incubated at 37°C for 16 h to activate immobilized proteases and stained with Coomassie blue. Type IV collagenolytic activity is indicated by transparent bands in the collagen-embedded gel (*arrowhead* on the *right*). *Left*, molecular weight standards (in thousands). These results were verified in a second experiment.

Table 2 *Effect of RDδ on invasive properties of MTLn3 cells in vitro: Invasion into chamber containing medium supplemented with 5% FBS*

	No. of cells/field (mean ± SD)			
Cell line	wt MTLn3	Vector	RDδ21	RDδ51
−Dox	343 ± 55[a]	395 ± 21	199 ± 54	270 ± 73
+Dox	346 ± 53	397 ± 21	<2	<2
% control[b]	101	101	<1[c]	<1[c]

[a] Values shown are the mean number of cells counted in four fields (×200) ± SD for $n = 6$ individual Transwells from two independent experiments.
[b] Percentage of invading cells in Dox-induced (+ Dox) cultures relative to control (− Dox) cultures migrating toward FBS.
[c] $P \leq 0.001$, by Student's t test.

static potential. To directly determine whether RDδ expression would alter MTLn3 cell tumor growth or metastases, wt and RDδ51 cells were transplanted into the mammary fat pad of female syngeneic rats. Where indicated, RDδ was induced by treating the cells in culture with Dox for 24 h before harvesting and/or by administering Dox to the rats through their drinking water (in culture/*in vivo*, Table 3). Animals were sacrificed on day 25, at which time primary tumors and lungs were harvested for further analysis. Macroscopic lung metastases are usually not apparent before day 25, although micrometastases may be observed earlier. The 25-day lag time between transplanting cells into the fat pad and identifying surface lung lesions reflects the time required for MTLn3 cells to migrate from the primary tumor site in the fat pad to the lungs and expand into a visible colony.

In experiment 1, we compared the effects of Dox on metastasis derived from wt and RDδ51 cells (Table 3A). Dox did not influence either the size of tumors or the number of metastases derived from wt cells, indicating that Dox treatment alone does not induce responses that interfere with MTLn3 tumor growth or metastasis (Table 3A). In the absence of Dox, tumor size and number of metastases for RDδ51 cells were not different from the wt cells. These results establish that neither Dox nor transfection *per se* significantly influence the metastatic potential of these cells.

In contrast to the wt cells, Dox treatment significantly decreased metastasis of the RDδ51 tumors (Table 3A). The number of lung lesions was reduced from a median of 140 to 3 in Dox-treated animals. Dox-induced inhibition correlated with Dox-induced HA-RDδ expression in the RDδ51 tumors (Fig. 5*A*). On the other hand, Dox did not influence RDδ51 tumor sizes, a result that is consistent with the observation that induced RDδ does not inhibit anchorage-dependent growth of cultured RDδ51 cells (Fig. 2). Thus, the main effect of RDδ is inhibition of metastasis rather than primary tumor growth.

In a second experiment, the mechanism of RDδ inhibition was investigated further by varying the Dox treatment intervals (Table 3B). We compared the effects of continual to partial (−3 to 14 or −3 to 7 days) Dox treatment on tumor size, metastasis, and RDδ expression. As in experiment 1, continual Dox treatment significantly inhibited metastasis without inhibiting growth of the mammary tumor (Table 3B and Fig. 5*B*). RDδ levels in day-25 tumors from the animals receiving the full course Dox treatment were high. In contrast, levels in day-25 tumors from animals receiving Dox treatment only up to day 7 or day 14 were reduced to the levels in tumors from animals that did not receive Dox, demonstrating that Dox-dependent RDδ induc-

Table 3 *Effect of RDδ expression on MTLn3 tumor growth and lung metastasis*

Cell line	Dox treatment In culture/*in vivo*[a]	Mammary tumor size at day 25 Mean diameter, mm	Lung metastases at day 25		
			Incidence	No. of lesions/lung Median (range)	Significance
A. Experiment 1: Effect of Dox treatment on wt and RDδ51 MTLn3 cells					
wt-MTLn3	− /−	26	4/4[b]	140 (31–250)	
	− /+	29	5/5	171 (91–250)	NSD[c]
	+ /−	27	5/5	– (250)[d]	NSD
	+ /+	19	5/5	128 (6–250)	NSD
RDδ51-MTLn3	− /−	24	5/5	140 (30–250)	NSD
	+ /+	23	3/5	3 (0–6)	$P < 0.005$
B. Experiment 2: Effect of varying Dox treatment intervals on RDδ51 tumor growth and metastasis					
	− /−	29	9/9	316 (179–452)	
	+/+ (−3 to 25)	24	7/9	7 (0–14)	$P < 0.005$
	+/+ (−3 to 14)	25	9/10	8 (0–16)	$P < 0.005$
RDδ51-MTLn3	+/+ (−3 to 7)	24	10/10	28 (2–54)	$P < 0.005$
	−/+ (7 to 25)	26	9/9	21 (5–36)	$P < 0.005$

[a] Where indicated, cultured cells were treated with Dox for 24 h before harvesting and transplanting into the mammary fat pad. Dox treatment of animals was initiated 3 days before cells were transplanted and continued for 25 (−3 to 25), 14 (−3 to 14), or 7 (−3 to 7) days. Alternatively, Dox treatment was not begun until day 7 after cells were transplanted (−/+, 7 to 25). All animals were sacrificed 25 days after injections. In preliminary experiments, we found no differences between wt and vector control cells. Therefore, to reduce the numbers of animals used, only one control cell type was used in each experiment. Studies with the cultured cells also demonstrated no significant differences between wt and vector control cell lines (see Table 2).
[b] Incidence, number of animals with visible lung lesions at necropsy (day 25)/total number of animals injected in the group.
[c] Statistical significance was determined by the Mann-Whitney Rank Sum test; NSD, no statistical difference for Dox-treated wt MTLn3 cells or uninduced RDδ51 cells compared with untreated parental MTLn3 cells. P indicates the confidence level for the statistical difference between Dox-induced (+/+) or (−/+) and control (−/−) RDδ51 cells.
[d] Lungs from all animals in this group had more lesions than could be counted and were scored ≥250.

tion is reversible *in vivo* (Fig. 5*A*). Despite the reduced RDδ levels, pulmonary metastasis were significantly decreased in these animals. This is likely to be due to the significant lag time between establishment and growth of a micrometastatic lesion to a visible colony. It is likely that the effect of Dox reversal would become more apparent at later times. However, the tumor burden at day 25 does not permit extending the experiments beyond this time.

In each of the protocols described above, cultured cells were pretreated with Dox before transplantation into the fat pad. Because Dox treatment inhibits cell attachment, inhibition of metastasis could be due to decreased attachment of injected cells in the mammary fat pad. However, because pretreatment with Dox does not significantly inhibit tumor growth, this does not seem likely. Nonetheless, to directly determine whether Dox pretreatment was necessary for the reduction in metastatic potential, RDδ51 cells that were not pretreated with Dox were transplanted into the fat pad of animals that were not primed with Dox. Dox treatment was begun on day 7, at which time a palpable tumor had formed (−/+, 7–25 days, Table 3B). Even under these conditions of delayed Dox treatment, RDδ expression significantly attenuated the development of metastatic lesions on the lungs (Table 3B). These results demonstrate that RDδ expression interferes with metastatic progression rather than establishment of the primary tumor. On the basis of the effects of RDδ expression on properties of cultured MTLn3 cells, it is likely that the reduced metastatic potential of Dox-treated RDδ51 cells is related to their decreased anchorage independence, invasion, motility, and/or substratum attachment.

In a second series of experiments, MT cells were directly injected into the tail vein, and lung metastases were evaluated 9 days later. In this assay, metastasis depends on the ability of the injected cells to survive in the circulation, escape from pulmonary capillaries, attach to lung tissue, and grow in that environment. All animals injected with control or Dox-treated wt cells produced large numbers of lung metastases (Table 4). Animals injected with uninduced RDδ51 cells also produced a large number of lung metastases, again indicating that uninduced RDδ cells are not significantly different from wt cells *in vivo*. In contrast to the wt cells, Dox-pretreated RDδ51 cells injected into syngeneic animals (with or without Dox treatment) formed relatively few metastatic lung lesions (+/− and +/+, Table 4). These experiments indicate that expression of RDδ during the first 24–48 h after injection critically influences survival in the circulation, attachment, invasion, and/or colonization of lung tissue. Thus, both the mammary fat pad and tail vein injection protocols indicate that the principal effect of RDδ expression *in vivo* is the suppression of lung colonization by MTLn3 cells.

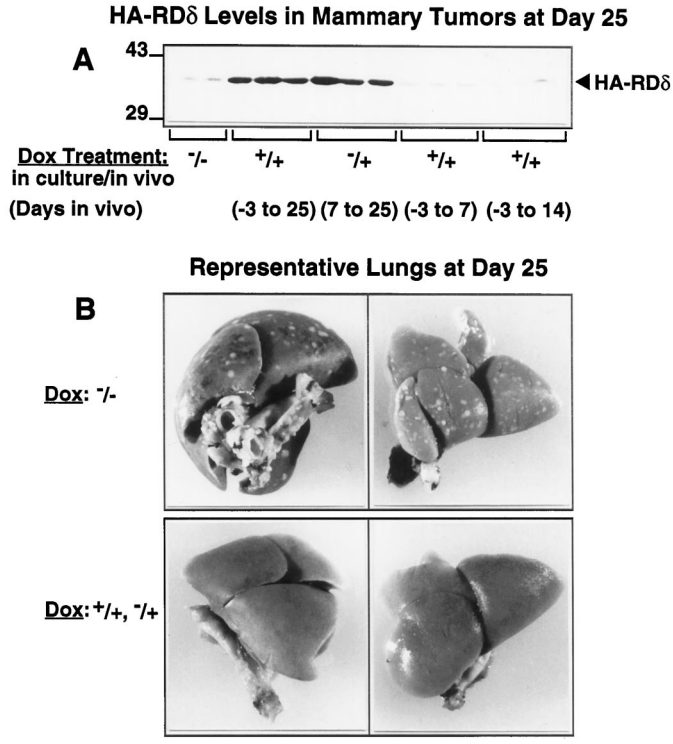

Fig. 5. Effect of Dox treatment duration on RDδ expression in mammary tumors and the development of pulmonary metastases. Where indicated by **(+),** Dox was administered to cells in culture, 24 h before injection into mammary fat pad, or was added to the drinking water from 3 days prior to injections (day −3) to the end of the experiment (day 25); actual initiation and duration of *in vivo* treatments are shown in parentheses for each group. On day 25, animals were sacrificed, and tissues were harvested. In *A*, tumor tissue extracts (50 μg of protein) were separated on SDS-PAGE and blotted to nitrocellulose; HA-tagged RDδ was detected by probing blots with HA-specific antibody (*arrowhead*). *Left*, molecular weight standards. In *B*, lungs harvested on day 25 were fixed in Bouin's solution, and surface lesions were counted using a dissecting microscope. The lungs shown are representative of the nine collected for the −/− and +/+ (day −3 to 25) groups.

Table 4 *Effect of Dox treatment and RDδ on the development of lung metastases derived from MTLn3 cells injected into the tail vein*

Cell line	Dox treatment in culture *in vivo*[a]	Lung metastases at day 9: Incidence	No. of lesions/lung Median (range)	Significance
Vector control	−/−	10/10[b]	85 (18–127)	
	+/−	11/11	67 (33–132)	NSD[c]
	−/+	9/9	93 (67–191)	NSD
RDδ51	−/−	10/10	72 (55–116)	NSD
	+/−	6/10	1.0 (0–9)	$P < 0.05$
	+/+	5/10	0.5 (0–2)	$P < 0.05$

[a] Control or Dox-treated vector control and RDδ51 MTLn3 cells were injected into the lateral tail vein of syngeneic rats. Where indicated, Dox was added to the animals' water supply. Animals were sacrificed 9 days after injection, and the number of macroscopic lesions/lung was determined with the aid of a dissecting microscope. Similar results were obtained in a second experiment. In preliminary experiments, we found no differences between wt and vector control cells. Therefore, to reduce the numbers of animals used, only one control cell type was used in each experiment. Studies with the cultured cells also demonstrated no significant differences between wt and vector control cell lines (see Table 2).

[b] Number of animals with metastases/total number of animals injected in group.

[c] Statistical significance was determined by the Mann-Whitney Rank Sum test. There were no statistical differences (NSD) between the median values for untreated versus Dox-treated vector control cells and untreated vector control cells versus untreated (−/−) RDδ51 cells. *P* indicates the confidence level for the statistical difference between Dox-induced and untreated RDδ51 cells.

Effect of RDδ on Cell Migration and Adducin Phosphorylation *in Situ*. The predicted mechanism of attenuation of metastases by RDδ *in vivo* is through inhibition of PKC substrate phosphorylation. *In vitro*, RDδ inhibits PKC catalytic activity and PKC binding to its substrates (27);[5] however, *in vivo* inhibition of substrate phosphorylation has not yet been demonstrated. We therefore monitored the effects of RDδ expression on the phosphorylation state of a known PKC substrate, adducin. In previous studies, we demonstrated that adducin phosphorylation was increased in cells induced to migrate into an artificial wound.[5] Wounding stimulates both cell growth and migration; therefore, aphidicolin was added to cultures to arrest cells in S-phase and minimize the effects of growth on our measurements of wound closure and adducin phosphorylation. Migration was assessed by measuring the decreased width of the scraped area in photomicrographs. Wound widths of vector control (± Dox) and RDδ51 cells without Dox treatment were similar, indicating that Dox treatment did not interfere with cell migration (Table 5). In contrast, Dox significantly increased wound widths of the RDδ51 cells, indicating that Dox-induced RDδ interfered with migration (Table 5). The contribution of cell growth to wound closure is apparent in the decreased wound width of cultures grown in the absence of aphidi-

[5] C. Chapline, J. Cotton, M. Tobin, Y. Zeng, and S. Jaken, manuscript in preparation.

Table 5 *Effect of RDδ expression on migration into an artificial wound*

	Mean width of wound (mm) ± SD			
	Vector control cells		RDδ51 cells	
	−Aphidicolin	+Aphidicolin	−Aphidicolin	+Aphidicolin
−Dox	1.1 ± 0.08[a]	1.4 ± 0.19	1.1 ± 0.06	1.4 ± 0.12
+Dox	1.0 ± 0.10	1.4 ± 0.20	1.9 ± 0.08	2.1 ± 0.10
Significance[b]	NSD	NSD	$P < 0.005$	$P < 0.005$

[a] Values shown are relative wound widths of triplicate cultures (mean ± SD) 18 h after scraping the cell monolayer with a sharp object. Data shown were derived from a single experiment, and similar results were obtained in a second experiment. Similar results were found with RDδ21 cells in one experiment.

[b] Significance for the difference between −Dox and +Dox conditions was determined by Student's *t* test; NSD, no statistical difference.

colin compared with cultures grown in the presence of aphidicolin (Table 5). These results indicate that RDδ expression inhibits migration and that the inhibitory effect of RDδ is independent of cell growth.

To determine whether the RDδ effects on cell migration could be due in part to the inhibition of substrate phosphorylation, phosphoadducin levels were monitored with an antibody that selectively recognizes adducins phosphorylated at their PKC-specific phosphorylation site.[5] pSer660-adducin has been used previously as a reporter to monitor endogenous PKC activity (16, 17). For these studies, 12 h after scrape wounding, cells were fixed and doubly stained with antibodies to detect phosphoadducin and HA-RDδ in the same cells. In the absence of Dox treatment, RDδ cells at the edge of the wound adopt a migratory morphology with characteristic leading lamellipodia that extended into the scraped area. Vector control cells, which looked identical, are not shown. pSer660-adducin staining is concentrated in the leading edge of the lamellipodia, although substantial cytoplasmic, perinuclear fluorescence is also observed (Fig. 6, *upper left*). Treatment with Dox significantly decreased pSer660-adducin staining intensity in RDδ21 cells (Fig. 6, *middle*). Furthermore, these cells did not display leading lamellipodia, consistent with the decrease in cell migration (Table 5). Identical results were seen with RDδd51 cells and are therefore not shown. The effects of RDδ are emphasized in high-passage RDδ21 cells that have become heterogeneous with respect to inducible RDδ expression (Fig. 6, *right*). Cells that no longer express RDδ have increased pSer660-adducin staining compared with RDδ-expressing cells in the same field. Treatment with Dox had no effect on pSer660-adducin levels in the vector control cells (data not shown), consistent with the results shown in Fig. 6, which demonstrate that Dox selectively affects phosphorylation in the RDδd-expressing cells within the heterogeneous RDδd21 population. Furthermore, leading lamellipodia can be seen in the nonexpressing cells, whereas these structures are not apparent in the RDδ-expressing cells. Low levels of phosphoadducin in RDδ-positive cells were not due to reduced adducin levels, because αa- and γg-adducin levels in lysates of cells cultured in the presence or absence of Dox did not differ by >15%, as determined by densitometry of immunoblots (data not shown). The fact that RDδ suppresses both Ser660-adducin phosphorylation and cell migration strongly suggests a functional link between PKCδ activity, adducin phosphorylation, and migration.

DISCUSSION

Mammary cancer is a disease in which events early in life, such as age of menarche and first pregnancy, dictate risk of disease decades later. Typically, early diagnosis correlates with favorable clinical outcome, indicating that tumor cells quickly progress to the metastatic phenotype. Clearly, a better understanding of processes that regulate the long latency period, promotion, and progression are needed to improve management of this disease. Progression from a nonmetastatic to a metastatic tumor cell involves a series of events that include changes in growth, gene expression, morphology, adhesion, and motility. PKC is known to regulate each of these events as well as tumor promotion in general. Thus, the purpose of this study was to test the hypothesis that the elevated PKCδ levels detected in highly metastatic MTLn3 cells contribute to the increased metastatic potential of those cells. One prediction of this hypothesis is that overexpressing PKCδ in less metastatic cells should promote their transformation. In fact, in a previous study, we demonstrated that expressing PKCδ in cells of moderate metastatic potential increased anchorage-independent growth (4). A second prediction is that down-modulating PKCδ activity in the highly metastatic cells should inhibit their malignant behavior. In the present investigation, we expressed the inhibitory RDδ fragment in the highly metastatic cells and demonstrated that RDδ inhibited transformation in cultured cells and lung metastasis in animals. These complementary studies point to an important role for PKCδ as a determinant of MT cell metastatic potential.

Expressing the regulatory domain of PKCδ to decrease endogenous PKCδ activity is somewhat different from most other studies that have used catalytically inactive PKCδ as a dominant-negative inhibitor (28–31). RDδ was used because recombinant RDs are very efficacious PKC inhibitors *in vitro* (27).[5] As shown in this study, expressed RDδ was also an effective PKC inhibitor in cells. Furthermore, studies with PKC regulatory/catalytic domain chimeras and regulatory domain fragments have demonstrated that the regulatory domains play a

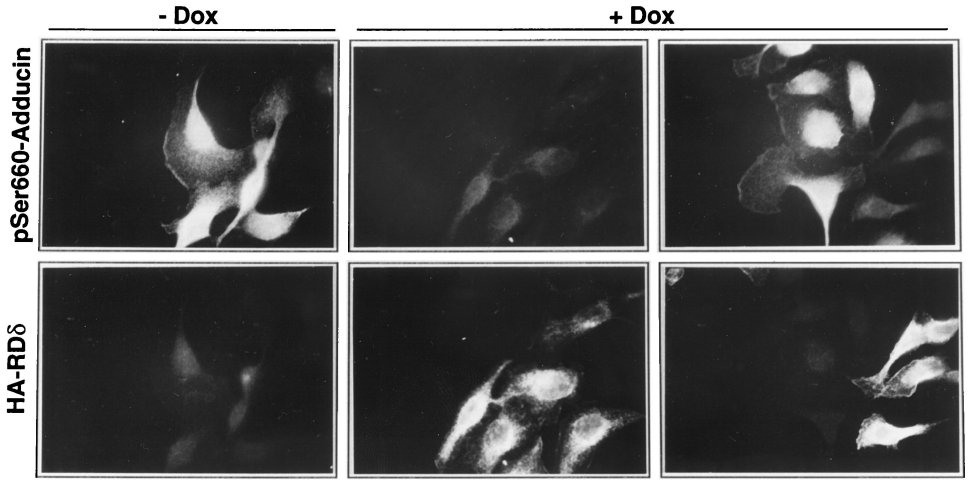

Fig. 6. Effect of RDδ expression on PKC-mediated adducin phosphorylation in migrating cells. MTLn3 vector control and RDδ21 cells that conditionally express RDδ were grown on coverslips to 80% confluence and were then cultured in the absence (*−Dox*) or presence (*+Dox*) of Dox for 24 h. An artificial wound was then scraped into each cell monolayer. Dox was maintained in cultures pretreated with Dox, and coverslips were incubated for 12 h. Cells were fixed and stained with anti-pSer660-adducin (+FITC-conjugated second antibodies) and anti-HA (+TRITC-conjugated second antibodies) to identify RDδ-positive cells in double-label immunofluorescence, as described in "Materials and Methods." Paired photographs were taken with a ×100 objective lens with the open wound on the *left side* of each photograph. The photographs shown represent the results obtained in four independent experiments.

role as isozyme-selective determinants of substrate recognition and subcellular targeting (32–36). Taken together, these results provide a firm rationale for using RD expression to inhibit endogenous PKCs, although further evidence is needed to establish the extent to which inhibition is isozyme selective.

The effects of RDδ on attachment, migration, and invasion all indicate that PKCδ and possibly other PKCs regulate cytoskeletal processes involved in cell adhesion and motility. Although other studies have implicated PKC activity in attachment and migration (37–39), our studies are the first to link PKCδ to these processes. We identified previously a group of cytoskeleton-associated PKC substrates called STICKs (reviewed in Ref. 13). Many STICKs are cytoskeleton-associated proteins, and inhibition of their phosphorylation establishes a mechanism by which RDδ can interfere with cytoskeleton-dependent processes, such as migration in cultured cells and metastasis *in vivo*. We studied adducin as an example of a STICK known to be involved in cytoskeletal remodeling. Adducins are barbed-end, actin-capping proteins, and PKC phosphorylation inhibits their actin-capping activity (40). Thus, localized phosphorylation would be predicted to result in localized uncapping and filament elongation, processes required for lamellipodia formation and migration. We found that in the absence of RDS expression, pSer660-adducin was concentrated in leading lamellipodia of migrating MTLn3 cells and induced expression of RDδ reduced pSer660-adducin levels. RDδ effects on cytoskeletal functions can, therefore, be linked to inhibition of phosphorylation of PKC substrates such as adducin and other STICKs.

It is important to note that Dox did not significantly decrease pSer660-adducin levels in primary tumors as determined by immunoblots (Fig. 5) and verified by immunohistochemical staining (data not shown). Histological sections of tumor tissue from both Dox-treated and control animals showed substantial pSer660-adducin levels, especially in cells at the tumor perimeter. Thus, it seems unlikely that decreased phosphorylation of substrates in the primary tumor accounts for the Dox-induced RDδ inhibition of metastasis. On the other hand, RDδ expression was also a potent inhibitor of metastasis of cells injected into the tail vein. To form a metastatic colony, these injected cells, as well as tumor cells that escape from the primary tumor, must survive in the circulation, extravasate, and attach to lung tissue. Because RDδ inhibits anchorage-independent growth/survival, attachment, and invasion *in vitro*, it seems likely that RDδ expression *in vivo* interferes with any or all of these processes rather than escape from the primary tumor.

Increased activity of other signaling pathways has also been linked to increased metastatic potential. In particular, increased rho, rac, and phosphatidylinositol 3-kinase activities have been linked to increased migration and invasion (41–43). Changes in levels of specific integrins or cadherins that regulate interactions with ECM or adjacent cells, respectively, have also been linked to increased metastatic potential (2, 3, 44). Our results indicate that PKCδ should be included among those pathways that regulate metastatic processes. Additional studies on PKCδ substrates and their functions will provide mechanistic insight as to how PKCδ regulates cytoskeleton-dependent processes associated with metastasis. Furthermore, properties of the substrates may also provide insight into how these different signaling pathways can converge on metastatic regulation. For example, recent studies have demonstrated that PKC, PKA, and rho-kinase phosphorylate adducin at different sites (45, 46). These distinct phosphorylation events differentially modulate adducin functions with regard to actin capping and polymerization. The net phosphorylation state of adducin at discrete subcellular sites is therefore a determinant of actin polymerization dynamics. In this way, adducin integrates signals from various pathways.

Our studies demonstrate that PKCδ signaling is an important determinant of metastatic potential in the 13762NF rat mammary metastasis model but do not yet address the general significance of PKCδ in human mammary or other cancers. Relatively little information on PKC isozyme levels in human mammary tissues is presently available. Monitoring PKC isozyme levels in human tissue samples is complicated by technical problems including the fact that PKCs can be rapidly degraded during sample collection. Furthermore, the relationship between PKC protein levels and endogenous activity is confused by the fact that active PKCs are down-regulated by proteolysis. Thus, in some cases, decreased PKC protein levels may actually correlate with increased activity (47). An alternative to monitoring PKC isozyme levels is to use phosphorylation state-selective antibodies to monitor substrate phosphorylation levels as reporters that reflect endogenous PKC activity levels. Immunohistochemical applications of these antibodies in prospective and retrospective studies of banked human tissues should be valuable for unraveling the role of PKC in human mammary tumor progression. Nonetheless, these initial studies in rat cells clearly establish a role for PKCδ in regulating cytoskeletal protein phosphorylation and cytoskeleton-dependent processes relevant to *in vivo* metastatic potential. Thus, PKCδ may be considered as a potential target for designing isozyme-selective synthetic inhibitors that could be used in combination with growth-limiting therapies to control the spread of metastases.

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