Protein Kinase Cε Mediates Polymeric Fibronectin Assembly on the Surface of Blood-borne Rat Breast Cancer Cells to Promote Pulmonary Metastasis*

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Malignant breast cancer cells that have entered the blood circulation from primary mammary fat pad tumors or are grown in end-over-end suspension culture assemble a characteristic, multi-globular polymeric fibronectin (polyFn) coat on their surfaces. Surface polyFn is critical for pulmonary metastasis, presumably by facilitating lung vascular arrest via endothelial dipeptidylpeptidase IV (CD26). Here, we show that cell-surface polyFn assembly is initiated by the state of suspension, is dependent upon the synthesis and secretion of cellular Fn, and is augmented in a dose- and time-dependent manner by plasma Fn. PolyFn assembly is regulated by protein kinase Cε (PKCε), which translocates rapidly and in increasing amounts from the cytosol to the plasma membrane and is phosphorylated. PolyFn assembly is impeded by select inhibitors of this kinase, i.e. bisindolylmaleimide I, Ro-32-0432, Gö6983, and Rottlerin, by the phorbol 12-myristate 13-acetate-mediated and time-dependent loss of PKCε protein and decreased plasma membrane translocation, and more specifically, by stable transfection of lung-metasstatic MTF7L breast cancer cells with small interfering RNA-PKCε and dominant-negative PKCε constructs (e.g. RD-PKCε). The inability to assemble a cell surface-associated polyFn coat by knockdown of endogenous Fn or PKCε impedes cancer cells from metastasis to the lungs. The present studies identify a novel regulatory mechanism for polyFn assembly on blood-borne breast cancer cells and depict its effect on pulmonary metastasis.

Fibronectin (Fn)2 is a “pro-metastatic” gene that is overexpressed in several malignancies (1–7) and, most prominently, in cancer cell lines selected for enhanced lung colonization (8–11). This pro-metastatic role of Fn is multifaceted, affecting several steps of the metastatic cascade by modulating cell adhesion, motility/invasion, cell cycle progression, and cell survival (for review, see Refs. 12–16). By analyzing cancer cells that had entered the blood circulation from malignant breast cancers implanted into the mammary fat pad of rats or mice, we discovered that blood-borne tumor cells were decorated with a unique, multiglobular coat of polymeric Fn (polyFn) (9, 17). PolyFn aggregates appeared to arise from focal accumulations of endogenous, cell surface-immobilized (“linearized”) Fn, which served as scaffolds for further Fn-self-assembly from Fn recruited from blood plasma (pFn) (9). Aggregates typically became increasingly deoxycholate-insoluble as they increased in size with time of incubation of suspended cancer cells in serum-containing medium in vitro. Biochemically, aggregates impressed as prominent, insoluble (covalently bonded) Fn polymers sitting on top of the stacks of SDS-polyacrylamide gels and, immunocytochemically, as large globules randomly dispersed over the entire cancer cell surface (9). This “cluster arrangement” of polyFn was shown to have the following functional implications. First, the conversion of Fn from the globular state of soluble Fn to the linearized state of insoluble, surface-associated Fn aggregates is associated with exposure of a novel, cryptic binding domain for the lung endothelial cell address in dipeptidylpeptidase IV (DPP IV) (9, 17–21). This DPP IV binding domain is present as a consensus motif in each of the 13th, 14th, and 15th type III repeats of Fn (17). Second, the DPP IV binding specificity for linearized (polymeric), but not for globular (soluble) pFn, allows tumor cell adhesion to endothelial DPP IV in the presence of high pFn concentrations (9). Third, the large Fn aggregates on cancer cell surfaces allow multiple binding interactions with endothelial DPP IV molecules, thereby generating adhesion strengths between cancer cells and endothelial cells that are able to withstand the rigors of hemodynamic shear stresses (9). The importance of the Fn/DPP IV-docking mechanism is substantiated by our discovery that synthetic peptides directed against the DPP IV binding domain in the 13th, 14th, or 15th type III repeats of Fn (17). Synthetic peptides encompassing the bulk of the extracellular domain of DPP IV dramatically impeded pulmonary metastasis in a rat breast cancer model (19, 20). These data are consistent with our finding that colonization of the lungs was greatly diminished in Fischer 344/CRJ rats, in which DPP IV is mutated causing a significantly decreased DPP IV protein expression in
pulmonary endothelia (21) as well as in DPP IV−/− mice (19). DPP IV−/− mice injected with lung-metastatic cancer cells lived significantly longer than their wild-type counterparts, an outcome granted by the formation of significantly fewer and smaller lung colonies.3

Although we have firmly established a critical dependence between the ability of the cancer cells to assemble an insoluble, globular polyFn-surface coat and lung colonization in an experimental metastasis model (9, 17), we still do not know whether blood-borne cancer cells use their own cellular Fn (cFn) or rely on ubiquitous pFn to assemble their polyFn surface coat, how cancer cells regulate the polyFn build-up, and how Fn surface deposits are transformed into covalently bonded, insoluble aggregates (9). To answer some of these questions we examined polyFn genesis in lung-metastatic MTF7L rat breast cancer cells subjected to end-over-end (EoE) suspension culture in serum- or pFn-containing medium, which together induce and augment the build-up of a polyFn surface coat similar to that observed on tumor cells that have entered the blood circulation (9). The data presented here show that assembly of the pre-metastatic, multiglobular polyFn surface coat on MTF7L breast cancer cells depends upon the synthesis and secretion of endogenous, cellular Fn (Fn1: EDA+EDB+IIICS120-Fn) and is regulated by membrane-translocation and Ser/Thr phosphorylation of protein kinase Cε (PKCε). Inhibitors of protein synthesis, protein secretion, and novel PKC isoforms (nPKCs) as well as transfection of MTF7L cells with the PKCε regulatory domain (RD) or PKCε siRNA species all substantially decrease the ability of the cancer cells to assemble a polyFn surface coat. Functionally, inability to assemble polyFn is associated with failure to colonize the lungs. Together, our studies provide novel insights of the regulation of Fn in suspended (blood-borne) breast cancer cells and provide a renewed appreciation for the previously recognized role of Fn in metastasis (1−7).

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Rabbit anti-PKCδ, -PKCε, -PKCθ, -PKCζ, and anti-hemagglutinin tag polyclonal antibodies and mouse anti-Fn (raised against a region in the human Fn-EDA domain; human- mouse-, and rat-specific) monoclonal antibody (anti-Fn[EDA]) were from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-Fn polyclonal antibodies that recognize pFns and cFns from both bovine and rat (anti-Fn[pan]; does not cross-react with fibrinogen, vitronectin, laminin, collagen type IV) was from Sigma, rabbit anti-PKCe polyclonal antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY), mouse anti-PKCe used for immunoprecipitation was from BD Biosciences, phycoerythrin (PE)-conjugated donkey anti-rabbit, PE-conjugated goat anti-mouse, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit, and HRP-conjugated goat anti-mouse antibodies were from Jackson ImmunoResearch (West Grove, PA), and rabbit anti-PKCa, -vinculin, and -actin were from Dr. Guan (University of Michigan). Pertussis toxin (Gαi inhibitor), PD98059 (MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2 inhibitor), SU6656 and PP2 (Src family kinase inhibitors), wortmannin and LY294002 (phosphatidylinositol 3-kinase inhibitors), JAK1 (Janus family kinase 1/2 inhibitor), Y27632 (ROCK1/2 inhibitor), U73122 (inhibitor of phosphatidylinositol-specific phospholipase C (PLC)), the PKC inhibitors calphostin C, Gö6976, HBDDDE, bisindolylmaleimide I (BIM I), Gö6983, BIM XI (Ro-32-0432), rottlerin, brefeldin A, monensin, and cycloheximide were from EMD Chemicals (San Diego, CA). Fetal bovine serum (FBS) was purchased from Gemini Bio-Products (Woodland, CA). Fn-free FBS (FFS) was generated by successive gelatin- and anti-Fn antibody affinity chromatography (22). All other chemicals and reagents were from Sigma.

**Cell Cultures—**MTF7L cells were derived from a lung metastasis generated by tail-vein injection of MTF7 breast cancer cells (obtained from Dr. D. R. Welch, University of Alabama at Birmingham, Birmingham, AL) into Fischer 344 rats. At an intravenous inoculation dose of 2 × 106 cells per rat, MTF7L cells consistently produce in excess of 400 lung colonies. Cells were grown in culture in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated FBS. For EoE suspension culture, MTF7L cells were grown to 80–90% confluence, then removed from the growth surface by trypsinization (0.25% trypsin, 0.02% EDTA in phosphate-buffered saline (PBS) for 10 min at 37 °C), washed twice in DMEM containing 10% FBS, and subjected to EoE suspension culture for 1 h (or as indicated) in 2-ml centrifuge tubes in DMEM plus 20% FBS at a concentration of 5 × 106 cells/ml (9, 17, 20). Tumor cells were used for all experiments within 10 passages from frozen stocks that were tested for metastatic performance immediately before freezing.

For metabolic labeling, MTF7L cells in logarithmic growth phase were labeled with [35S]methionine (0.33 mCi/3 ml) in methionine-free DMEM (both from MP Biomedicals, Solon, OH) containing 20 μM methionine and 10% dialyzed, Fn-free FBS as previously described (9). For 32P labeling, cells were serum-starved overnight, then incubated for 4 h in phosphate-free DMEM containing 100 μCi/ml [32P]orthophosphate (ICN Biochemicals, Irvine, CA) and washed in 3 changes of PBS. Labeled cells were subjected to EoE suspension culture as describe above and immediately processed for biochemical analyses.

**Plasmid Constructs, Transfection, and Selection—**The constructs wtPKCe, RD-PKCe, and RD-PKCζ cloned into pEGFP-N1 were obtained from Dr. C. Larsson (Lund University, Malmö, Sweden) (23), and wtPKCd and RD-PKD were cloned into pcDNA3.1 from Dr. D. Mayer (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (24). For siRNA knockdown of protein expression, the following nucleotide (nt) sequences were cloned into pRNA6-hygro vector (GenScript, Piscataway, NJ): sequence 1 (5′-atgcatgcagtagctgctg-3′ (NM_019143.1, nt 681−699)) and sequence 2 (5′-aaacaacctgctgccggag-3′ (NM_019143.1, nt 4452−4472)) (25) for rat Fn1; sequence 3 (5′-aatctgccagtaacg-3′ (NM_017171.1, nt 194−211) (26) and sequence 4 (5′-aagtcttgagctgctgc-3′ (NM_017171.1, nt 1603−1621)) for rat PKCe; sequence 5 (5′-aactgcagcatagcactgct-3′) for the nonspecific control sequence. The siRNA plasmid pKD-PKCa-v6 was purchased

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3 H. C. Cheng, unpublished data.
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from Upstate. All plasmid constructs were verified by double-stranded sequencing.

MTF7L cells grown to 70% confluence were transiently transfected with the above vector constructs or vector alone using Lipofectamine Plus as described by the manufacturer (Invitrogen). Transfection rates assessed by expression of GFP that is either tagged to the cDNA of interest or co-transfected at a ratio of 1:50 with the cDNA of interest were 20–30%. Cells were used in the various assays 48 h after transfection unless otherwise stated. Stable clones were obtained by hygromycin selection (750 μg/ml). In some cases hygromycin-selected clones (cl) were further selected by fluorescence-activated cell sorting (FACS) for optimal expression.

Flow Cytometry—FACS was used to quantify Fn expression on MTF7L breast cancer cell surfaces (9, 17). Tumor cells that had been subjected to EoE suspension culture were washed twice in DMEM containing 1% bovine serum albumin (BSA), then incubated with rabbit anti-Fn[pan] antibody diluted 1:100 in PBS containing 1% BSA (PBS-BSA) for 1 h at 4 °C. After washing in PBS-BSA, tumor cells were stained with PE-conjugated donkey anti-rabbit antiserum in PBS-BSA for 1 h at 4 °C and fixed in 2% paraformaldehyde in PBS. In select experiments cells were stained with mouse anti-Fn[EDA] (diluted 1:50) and PE-conjugated goat antimouse antiserum. FACS analysis was performed on a Coulter Epics Profile (Coulter Electronics, Hialeah, FL). Non-specific fluorescence was accounted for by incubating tumor cells with non-immune rabbit serum instead of primary antibody. To quantify the effect of overexpressed or knocked-down proteins on polyFn assembly, we generate bivariate distributions of red fluorescence (y axis: cells stained with anti-Fn antibodies and PE-conjugated secondary antibodies) and green fluorescence (x axis: same cells expressing GFP-tagged protein or co-transfected with GFP and cDNA of interest). The levels of polyFn expression in the cell population that emitted high GFP fluorescence were taken as a reflection of the effect of the transfected cDNA on polyFn assembly. To assess the effect of inhibitors of cell signaling, tumor cells were incubated with inhibitor 30 min before (adherent) and throughout EoE suspension culture, then subjected to polyFn quantification as described above. Controls were tumor cells incubated in equimolar inhibitor solvent concentration.

Semi-quantitative Reverse Transcription-PCR Analyses—Total RNA was prepared from MTF7L grown as adherent monolayers or in EoE suspension cultures by extraction with Trizol as described by the manufacturer (Invitrogen). For every experimental sample, total RNA was quantified both spectrophotometrically and electrophoretically, and amounts were adjusted so that 1 μg was reverse-transcribed (SuperScript reverse transcriptase; Invitrogen). cDNA was subjected to PCR (93 °C for 30 s; 55 °C, 30 s; 72 °C, 30 s; 35 cycles) using TaqDNA polymerase (Invitrogen) and primer sets derived from rat Fn1 (NM_019143), PKCδ (NM_133307), PKCe (NM_017171), PKCγ (NM_031085), PKCθ (XM_341553), and PKCζ (NM_022507). Controls were run in the absence of reverse transcriptase. Glyceraldehyde-3-phosphate dehydrogenase served as reference standard.

Cell Fractionation—MTF7L cells and transfectants thereof were incubated in EoE suspension culture in DMEM containing 20% FBS for the indicated periods of time. Cells were washed in PBS, collected by centrifugation, and resuspended in 0.5 ml ice-cold Tris buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 0.1 mM NaVO₃, 20 μM leupeptin, 0.1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted by two 15-s cycles of sonication at 4 °C using a microprobe sonicator at maximum power. After removal of unbroken cells and nuclei by centrifugation (500 × g for 5 min at 4 °C), supernatants were centrifuged at 16,300 × g for 15 min at 4 °C. The resulting supernatant was designated the cytosolic fraction. The pellet was solubilized in 0.5 ml of buffer A containing 1% Triton X-100 for 1 h at 4 °C EoE, then centrifuged at 16,300 × g for 15 min at 4 °C. The detergent-soluble fraction was designated the membrane fraction. Twenty micrograms of protein from both the cytosolic and membrane fractions were separated by SDS-PAGE (10%), transferred to nitrocellulose membrane at 4 °C, and probed by Western blotting as described (9).

Cell Lysis, Immunoprecipitation, Western Blotting, and Autoradiography—Cells were extracted with lysis Tris buffer containing 1% Triton X-100 for 1 h at 4 °C (9). Total cell lysates or cytosolic and membrane fractions were subjected to (i) SDS-PAGE (~20–50 μg of protein) and Western blotting using anti-Fn[pan], anti-Fn[EDA], or various PKC isoform-specific antibodies, horseradish peroxidase-conjugated donkey anti-rabbit, or goat anti-mouse secondary antibodies and ECL for detection of bound antibody as described (9) and (ii) immunoprecipitation with anti-Fn[pan], anti-PKCδ, or anti-PKCζ antibodies (27). Immunoprecipitates obtained from lysates of unlabelled, [32P]orthophosphate-, or [35S]methionine-labeled cells were separated by SDS-PAGE (6–12% polyacrylamide) and analyzed by autoradiography (radio-labeled samples) or blotted to nitrocellulose membranes and probed with either anti-Fn[pan], anti-Fn[EDA], anti-PKC isoform-specific antibodies, or anti-Ser(P) and anti-Thr(P) antibodies (9).

Tumor Cell Proliferation Assay—MTF7L cells and clones thereof were seeded into 96-well microtitration plates (500 cells/well) and incubated in DMEM containing 10% FBS. Cell growth was monitored in daily intervals for up to 4 days. At the end of each incubation period, tumor cells were fixed with 2% paraformaldehyde in PBS and then stained with 0.5% crystal violet in 20% methanol as described (21). Absorbance was read on a microplate reader (Bio-Tek Instruments) at 562 nm and graphed as a function of time of incubation.

Isolation of Blood-borne Cancer Cells from Tumor-bearing Rats—MTF7L cancer cells (1 × 10⁶ cells/50 μl of DMEM) were injected into the 4th (left + right) mammary fat pads of six 6-week-old female Fischer 344 rats. At a tumor diameter of ~2 cm, rats were anesthetized by intraperitoneal injection of sodium pentobarbital (65 mg/kg body weight), and blood was collected by cardiac puncture. Poolled, EDTA-treated blood was transferred to precooled 50-ml centrifuge tubes containing 15 ml of OncoQuick tumor enrichment medium below a porous barrier (Greiner Bio-One, Longwood, FL) and centrifuged at 1600 × g for 20 min at 4 °C in a swing-out rotor as

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the number of lung colonies, and the colony diameters were determined for each cell variant (21).

RESULTS

Endogenous Fn Is Required for PolyFn Assembly on Suspended Breast Cancer Cells—Breast cancer cells entering the blood circulation assemble a characteristic surface coat of globular polyFn as evidenced by anti-Fn(pan) staining of cancer cells isolated from the blood of mammary tumor-bearing rats (Fig. 1A). This phenomenon is mimicked when cancer cells are grown in EoE suspension culture in the presence of serum (9). To examine whether polyFn assembly is mediated by exogenous pFn alone or whether it critically depends on the synthesis of endogenous cFn, we incubated MTF7L breast cancer cells, which express high levels of Fn1 (EDA+EDB+CSIII120) in EoE suspension culture in DMEM containing either 20% complete FBS or FFS. FFS-treated tumor cells stained with either anti-Fn(pan) (Fig. 1B) or anti-Fn[EDA] (Fig. 1B, inset) exhibited similar numbers of polyFn globules on their surfaces as FBS-treated cells (Fig. 1C). However, the addition of complete FBS to the suspension medium significantly augmented the size of individual polyFn globules relative to those generated by FFS (Fig. 1, B and C). The serum effect on polyFn assembly appeared to be mediated only in part by pFn as shown by FACS analysis of MTF7L cells incubated in EoE suspension culture in the presence of (i) 20% FBS (Fig. 1, D and E), (ii) 6 μg/ml pFn (amount present in 20% FBS; 22), or (iii) 20% FFS (Fig. 1E). Both 20% FFS and pFn (6 μg/ml) were significantly less effective in promoting polyFn assembly than FBS (Fig. 1E). The potency of FFS to promote polyFn assembly could be restored to that of FBS by the addition of 6 μg/ml pFn (Fig. 1E, inset). Thus, FFS appeared to promote surface deposition of cellular Fn (Fn1) on suspended MTF7L cells, whereas pFn in a time- and dose-dependent manner contributed to augmentation of the Fn polymers by Fn-Fn self-assembly (Fig. 1F).

To further test the hypothesis that surface deposition of a scaffold of endogenous cFn was important for the initiation of polyFn assembly, we examined whether inhibitors of protein synthesis (e.g. cycloheximide) (29) or secretion (e.g. monensin, brefeldin A) (30, 31) would affect polyFn assembly on sus-

Lung Colony Assays—To determine the effects of Fn or PKCe knockdown on the lung colony efficiency, selected MTF7L clones as well as wt or vector-transfected MTF7 cells (2 × 10^6 cells/0.3 ml DMEM/rat) were injected into the lateral tail vein of 5-week-old female Fischer 344 rats (5–9 rats/experiment) as described (9, 21). Rats were sacrificed 30 days after tumor cell injection. Means and standard deviations of the lung weights, described by Rosenberg et al. (28). After a second round of centrifugation of the fluid in the upper compartment, cells were washed with PBS-BSA and stained with anti-Fn antibodies as described above. Tumor cells were readily differentiated from contaminant blood mononuclear cells by size and intensity of anti-Fn staining in comparison with preparations from MTF7L cell-spiked blood.

PolyFn globules expressed on the surface of suspended (blood-borne) MTF7L breast cancer cells. A, MTF7L breast cancer cells were isolated from the blood of tumor-bearing rats using the OncoQuick tumor enrichment medium. The mononuclear cell fraction separated from the blood cells, platelets, and polymorphonuclear leukocyte fractions was washed several times in PBS, then stained with rabbit anti-Fn(pan) antibodies followed by PE-conjugated donkey anti-rabbit IgG antibodies and observed under a fluorescent microscope. There are multiple Fn globules randomly dispersed over the cancer cell surface. B and C, MTF7L cells subjected to EoE suspension culture in DMEM + 20% FFS (B) or DMEM + 20% complete FBS (C). Both are incubated for 1 h at 37 °C, then stained as described under A. Note that the same numerical density of Fn stippled are observed in B and C, but a larger stipple size is present in C, mimicking those observed in A, D, inset, same treatment, but stained with anti-Fn[EDA]. D, FACS analysis of MTF7L cells incubated EoE for 1 h in DMEM + 20% FBS and stained with anti-Fn[pan] antibodies as described under A. E, FACS analysis of MTF7L cells incubated EoE for 1 h in DMEM + 6 μg/ml pFn (green), DMEM + 20% FFS (red), or DMEM + 20% FBS (black). Notice that tumor cells treated with DMEM + 20% FBS (containing –6 μg/ml pFn) exhibit a higher mean fluorescence than tumor cells treated with DMEM + 20% FF or DMEM + 6 μg/ml pFn. E, inset, tumor cells incubated with 20% FFS + 6 μg/ml (red) and 20% FBS (green) show identical polyFn assembly. F, FACS analysis of MTF7L cells treated for the indicated periods of time with DMEM + 20% FBS. There is a time-dependent increase in polyFn assembly. Bars in A–C, 50 μm.

FIGURE 1. PolyFn globules expressed on the surface of suspended (blood-borne) MTF7L breast cancer cells. A, MTF7L breast cancer cells were isolated from the blood of tumor-bearing rats using the OncoQuick tumor enrichment medium. The mononuclear cell fraction separated from red blood cells, platelets, and polymorphonuclear leukocyte fractions was washed several times in PBS, then stained with rabbit anti-Fn(pan) antibodies followed by PE-conjugated donkey anti-rabbit IgG antibodies and observed under a fluorescent microscope. There are multiple Fn globules randomly dispersed over the cancer cell surface. B and C, MTF7L cells subjected to EoE suspension culture in DMEM + 20% FFS (B) or DMEM + 20% complete FBS (C). Both are incubated for 1 h at 37 °C, then stained as described under A. Note that the same numerical density of Fn stippled are observed in B and C, but a larger stipple size is present in C, mimicking those observed in A. D, inset, same treatment, but stained with anti-Fn[EDA]. D, FACS analysis of MTF7L cells incubated EoE for 1 h in DMEM + 20% FBS and stained with anti-Fn[pan] antibodies as described under A. E, FACS analysis of MTF7L cells incubated EoE for 1 h in DMEM + 6 μg/ml pFn (green), DMEM + 20% FFS (red), or DMEM + 20% FBS (black). Notice that tumor cells treated with DMEM + 20% FBS (containing –6 μg/ml pFn) exhibit a higher mean fluorescence than tumor cells treated with DMEM + 20% FF or DMEM + 6 μg/ml pFn. E, inset, tumor cells incubated with 20% FFS + 6 μg/ml (red) and 20% FBS (green) show identical polyFn assembly. F, FACS analysis of MTF7L cells treated for the indicated periods of time with DMEM + 20% FBS. There is a time-dependent increase in polyFn assembly. Bars in A–C, 50 μm.
nous Fn, lung colonization, assessed by the averages of lung weights, colony numbers, and colony diameters, was significantly decreased relative to wtMTF7L cells or tumor cells that were stably transfected with an unspecific nucleotide sequence (Fig. 4, siFn-cl1).

**PolyFn Assembly Occurs in a PKC-dependent Manner**—To identify the signaling cascade that regulates polyFn assembly, we screened a diverse group of inhibitors of cell signaling for their ability to impede polyFn assembly as determined by routine FACS analyses of anti-Fn[pan]-stained MTF7L breast cancer cells subjected for 1 h to EoE suspension culture in DMEM + 20% FBS containing 20% FBS.

**FIGURE 2. Inhibitors of protein synthesis and secretion prevent polyFn assembly.** Adherent MTF7L rat breast cancer cells were exposed for 30 min to 10 μg/ml cycloheximide (A), 1 μM monensin (B), or 5 μM brefeldin A (C), then subjected to EoE suspension cultured in DMEM + 20% FBS in the presence of the respective drug or drug solvent (S). After a 1-h incubation period, cells were stained with anti-Fn[pan] antibodies as described in Fig. 1A (control), MTF7L cell stained with non-immune rabbit IgG (rlgG). Tumor cells treated with cycloheximide, monensin, and brefeldin A exhibit a dramatic reduction in the cell surface-associated polyFn, relative to those treated with drug solvent.

**FIGURE 3. Effect of siRNA knockdown of cFn on polyFn assembly in the presence of 20% FBS.** Hygromycin-resistant clones were selected from MTF7L breast cancer cells transfected with the rat Fn1 nt sequences 1 (clones cl1 and cl3), rat Fn1 nt sequence 2 (clone cl2), or unspecific nt sequence 5 (multi-clone mclus), all cloned into pRNAU6-Hygro for siRNA targeting as described under “Experimental Procedures.” A, anti-Fn[pan] Western blot from lysates of siFn clones cl1 and cl3 and the control clone mclus. B, MTF7L cells metabolically labeled with [35S]methionine as described under “Experimental Procedures” and Cheng et al. (9) were subjected to EoE culture in DMEM + 20% FBS for 4 h, then extracted in lysis buffer containing 1% Nonidet P-40. Lysates were immunoprecipitated with anti-Fn[pan], and immunoprecipitated proteins were separated by SDS-PAGE (6%) under non-reducing conditions. Notice the significant reduction in the amount of anti-Fn[pan] immunoprecipitable material (polyFn) residing on top of the stacking gel. FACs analysis of MTF7L siFn clones cl1, cl2, and cl3 (green) and the control clone mclus (black, control), cultured and stained with anti-Fn[pan] as described in Fig. 1A. Mc, mean fluorescence of multiclone mclus (black); Mpolyn, mean fluorescence of siFn clones cl1, cl2, and cl3 (green). FL2-H, FACs phycocyanin channel peak emission value. D and E, micrographs from MTF7L siRNA-Fn clone cl1 (D) and control-cln mclus (E), both processed and stained with anti-Fn[pan] as described in Fig. 1A. Notice a significantly decreased polyFn assembly in MTF7L siFn clone cl1 (C) relative to MTF7L control clone mclus (D) even though both clones (D and E) were incubated with DMEM + 20% FBS for the same period of time (1 h at 37 °C) in EoE suspension culture. Bars in D and E, 50 μm.
with Fn-matrix assembly and stress fiber formation in adherent cells (32, 33), steadily decreased during the polyFn assembly phase on suspended breast cancer cells. Moreover, transfection with constitutively active Rho (RhoAQ63L) suppressed polyFn assembly, whereas transfection with dominant negative Rho (RhoAT19N) appeared to promote polyFn assembly.4 In contrast, polyFn assembly was dramatically reduced by U73122, an inhibitor of PLC (34), and the PKC inhibitor calphostin C, which competes with the diacylglycerol and phorbol ester binding site of conventional PKCs (cPKCs) and nPKCs (35) (Fig. 5A). Together, these findings suggest involvement of a cPKC or nPKC isoform but not an atypical PKC isoform, presumably acting downstream of PLC in polyFn assembly on MTF7L cancer cell surfaces (36).

**PKCe is the Mediator of PolyFn Assembly and Metastasis—**

Our attempts to identify the PKC isoform responsible for polyFn assembly were preceded by determining the c- and nPKC expression levels in MTF7L breast cancer cells. As reported for adherent, metastatic mammary tumor variants by Kiley et al. (37), suspended MTF7L cells strongly express PKCa, PKCb, and PKCe, both at the mRNA and protein levels (Fig. 5B). PKCη mRNA is expressed moderately and protein weakly, and PKCθ mRNA is expressed weakly and protein non-detectably (Fig. 5B). Next, we used select PKC inhibitors to narrow the spectrum of PKC isoforms involved in polyFn assembly. Failure of high doses of the cPKC inhibitors Gö6976 and HBBDE (38) to prevent polyFn assembly ruled out participation of PKCa in polyFn assembly (Fig. 6A). In contrast, inhibitors that in addition to cPKCs also inhibited nPKCs, including BIM I, Gö6983, BIM XI (Ro-32-0432), and rottlerin (39–43), decreased polyFn assembly in dose-dependent manners (Fig. 6B). Albeit these inhibitor data do not provide conclusive evidence of the nPKC isoform involved in polyFn assembly, PKCα, PKCβ, and PKCε are our most likely candidates for involvement in polyFn assembly based on high protein expression levels and inhibitor activity.

To examine the roles of PKCβ, PKCe, and PKCη (nPKC isoforms previously associated with breast cancer metastasis, see Refs. 44–46) in polyFn assembly, we transfected MTF7L cells with the RDs of these isoforms or knocked down their expression by PKC isoform-specific siRNA oligonucleotides. The RDs of PKCβ (co-transfected with GFP at a ratio of 1:50), PKCe, and PKCε-GFP were transiently expressed in MTF7L breast cancer cells. Forty-eight hours after transfection, cancer cells were subjected to EoE suspension culture in serum-containing medium for 1 h, then stained with anti-Fn antibodies, and Fn was visualized by PE-conjugated secondary antibodies.

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4 H. C. Cheng and B. U. Pauli, manuscript in preparation.
Cells were analyzed by bivariate, dual-color (red-green) FACS. Among the three nPKC isoforms, only RD-PKCe inhibited polyFn assembly, as depicted by a significant downshift in the number of GFP-positive cells that strongly stained with anti-Fn antibody, i.e., only 21% of tumor cells that expressed high levels of polyFn also expressed high levels of polyFn, relative to 56% in vector-transfected cells (Fig. 7A). The RD sequences of PKCe had no effect on polyFn assembly, whereas the RD sequence of PKCζ caused an increase in the number of strongly GFP-positive cells that expressed high levels of polyFn (Fig. 7A), suggesting that PKCζ might exert a negative regulatory role in polyFn assembly.

A similar effect on polyFn assembly to that of RD-PKCe and RD-PKCe was observed when MTF7L cells were co-transfected transiently with GFP and siRNA-PKCe or siRNA-PKCe and subjected to bivariate FACS analyses 48 or 72 h after transfection. Again, knockdown of PKCe suppressed polyFn assembly, whereas knockdown of PKCζ resulted in increased polyFn assembly on MTF7L cell surfaces (Fig. 7B). These results were confirmed by the analysis of stable MTF7L clones with “siRNA knockdown” of PKCe. PKCe knockdown resulted in a significant decrease in polyFn assembly relative to a control clone transfected with an unspecific siRNA nucleotide sequence (Fig. 8A). As expected, the FACS-measured decrease in polyFn paralleled decreased PKCe protein expression (Fig. 8B) as well as decreased amounts of polyFn residing on top of the stack of polyacrylamide gels (Fig. 8C, top), generated from anti-Fn[pan] immunoprecipitates of metabolically labeled siRNA-PKCe and control clones. The biochemically identified decrease in polyFn was reflected in decreased surface association of polyFn on MTF7L cells stained with anti-Fn[pan] (Fig. 8C, bottom). Functionally, impaired polyFn assembly in siRNA-PKCe clones is associated with a dramatic decrease in lung colonization (Fig. 4).

**DISCUSSION**

The role of Fn in cancer progression is controversial. Early studies suggest that Fn may act as a tumor suppressor gene, promoting differentiation and suppressing proliferation, migration, invasion, and metastasis (for review, see Refs. 13–16). In accordance, expression of pFn in Fn-negative LMM3 murine mammary cancer cells reduced the rate of migration as well as spontaneous and experimental metastasis (47) and forced expression of the α5β1 integrin, which was thought to capture secreted cFn at the cell surface, convert it into fibrils, and then deposit it in the extracellular matrix, reduced motility, and tumorigenicity of transfected tumor cells (48). These findings are contrasted by cDNA or oligonucleotide microarray
analyses that attempted to identify genes whose expression was associated with malignant behavior of breast cancers. In several of these studies, Fn (Fn1, EDA+/EED+/ IIICS120) was found to be consistently overexpressed in invasive/metastatic breast tumors in toto as well as in tumor cells isolated by laser capture microdissection from a primary invasive ductal carcinoma and an auxiliary node harboring metastatic breast cancer (5–7). Interestingly, ErbB2 overexpression, observed in 25–30% of invasive ductal breast carcinomas and correlated with a poor clinical prognosis, was associated with Fn1 overexpression in 11 of 36 cases (49). Overexpression of Fn was also noticed in cancer cell lines that exhibited an invasive/metastatic phenotype, including MDA-MB-231, MeWo-70W, PC3-ML, 4T1, LLC1, B16-F10, K7M2, MTF7, and RPC-2 (17) and was most dramatic in cancer cell lines that were subjected to a selection process for enhanced lung metastatic performance (9, 11, 17) or in highly metastatic clones derived from a rhabdomyosarcoma (8) or B16 melanoma (10) but not in non-metastatic clones (8). Moreover, expression of the metastasis suppressor gene nm23 in MDA-MB-435 breast cancer cells resulted in a down-regulation of genes associated with adhesion and motility, including Fn1 (50). On the other hand, neither the poor prognosis signature obtained by large-scale transcript profiling of human breast cancers (51, 52) nor the gene expression profile that was associated with lung metastasis of selected MDA-MB-231 clones (i.e., lung-metastatic signature) contained Fn (53). A possible explanation for this result may be related to the multifunctional role of Fn in tumor progression, requiring high levels of Fn expression throughout tumor progression. For example, in the early stages of progression Fn promotes survival, cell cycle progression, angiogenesis, and in later stages Fn promotes motility/invasiveness and dissemination and implantation in sec-

**FIGURE 7.** A, effect of RD-PKCe, RD-PCe, and RD-PKCη on polyFn assembly. MTF7L breast cancer cells grown to a density of 70–80% were transiently transfected with the RD of PKCe, PKCη, and PKCe as described under “Experimental Procedures.” Forty-eight hours after transfection, cells were subjected to EoE suspension culture in DMEM + 20% FBS for 1 h at 37°C, then stained with anti-Fn[pan] antibodies as described in Fig. IA and immediately analyzed by bivariate, dual-color (red fluorescence (FnF) versus green fluorescence (GFP-F)) FACS. Gates were selected based on the staining of wtMTF7L cells with non-immune rabbit IgG and Fn staining of vector-transfected MTF7L cells. Cells in the upper right quadrant of the scatter gram (=strong expressers of RD-PKC and Fn) were expressed as percent of the sum of cells in the right upper and lower quadrants (=strong RD-PKC expressers, gray area), thereby providing a quantitative measure of the inhibitory activity of each RD-PKC isoform on polyFn assembly. Data show inhibitory activity of RD-PKCe, stimulatory activity of RD-PKCe, and no effect of RD-PKCη. B, effect of PKCe and PKCe siRNA knockdown on polyFn assembly; MTF7L breast cancer cells grown to a density of 70–80% were transiently co-transfected with either pRNAU6-Hygro-siRNA-PKCe (sequence 3), pKD-PKCe-v6, or pRNAU6-HygrosiRNA-us (unspecific nt sequence 5) and GFP at a cDNA ratio of 50:1 as described under “Experimental Procedures.” Forty-eight hours after transfection, tumor cells were analyzed by FACS. Using a similar gate setting as in A (MTF7L cells stained with non-immune rabbit IgG and MTF7L cells transfected with siRNA-us and stained with anti-Fn[pan] antibodies), only 33% of the siPKCe transfectants exceeded the ~50% threshold in polyFn assembly of siRNA-US transfectants, whereas in siPKCe-transfectants this value was 73%.
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FIGURE 8. Analysis of stable clones selected from siRNA-PKCe-transfected MTF7L cells. siRNA-PKCe clones (cl1 and cl2) were hygromycin-selected from MTF7L cells transfected with nt sequence 3, siRNA-PKCe-cl1 from MTF7L cells transfected with the nt sequence 4, and control clone mclus from MTF7L cells transfected with the unspecific nt sequence 5 (see “Experimental Procedures”). A, clones PKCe-cl1 and -cl2, and control clone mclus subjected to EoE suspension culture in DMEM + 20% FBS for 1 h were stained with anti-Fn[pan] and analyzed by FACS. PolyFn assembly by MTF7L clones PKCe-cl1 and PKCe-cl2 was significantly decreased relative to the mclus control clone. B, Western blot analysis for PKCe and vinculin expression in lysates from siPKCe-cl1, -cl2, and -cl3, and the mclus clone. PKCe protein is decreased in siPKCe clones relative to the unspecific siRNA-mclus control clone. C, lysates from MTF7L clones mclus and siPKCe-cl1 labeled with [35S]methionine and processed as described in Fig. 3 were subjected to anti-Fn[pan] immunoprecipitation followed by SDS-PAGE and autoradiography. There is a significant decrease in polyFn residing on top of the stacking gel in siPKCe-cl1 relative to the mclus control, mimicking the degree of tumor cell surface-associated polyFn of these clones stained with anti-Fn[pan] (C, bottom).

FIGURE 9. Membrane translocation and phosphorylation of PKCe. A, PKCe translocates in increasing amounts from the cytosol to the plasma membrane during 0, 15, 30, and 60 min of EoE suspension culture of MTF7L in DMEM + 20% FBS. Notice a slightly higher molecular weight of PKCe harvested from the membrane fraction (M) relative to that from the cytosolic fractions (C). B, time course of PKCe phosphorylation. MTF7L were incubated in EoE suspension culture for the indicated periods of time in DMEM + 20% FBS in the presence or absence of BIM XI (500 nM). At the end of the indicated time periods, cells were lysed, and lysates subjected to anti-PKCe immunoprecipitation. Precipitates were probed by Western blotting with anti-PKCe, anti-Ser(P), and anti-Thr(P) antibodies. Maximal phosphorylation of PKCe was observed at 30 min of EoE suspension culture. Thereafter, the PKCe phosphorylation decreased to barely detectable levels at 60 min. PKCe of MTF7 cells treated with BIM XI stained weakly or non-detectably with anti-Ser(P) and anti-Thr(P). C and D, [32P]orthophosphate incorporation into PKCe (C) and PKCe (D). MTF7 cells labeled for 60 min in phosphate-free DMEM containing 100 μCi/ml [32P]orthophosphate as described under “Experimental Procedures” were subjected for 30 min to EoE suspension culture in DMEM + 20% FBS (lane 2), DMEM + 20% FBS (lane 3), and DMEM + 20% FBS + 500 nM BIM XI (lane 4) (control). [32P]Orthophosphate-labeled, adherent cells incubated for 30 min in DMEM + 20% FBS (lane 1). Anti-PKCe (C) and anti-PKCe (D) immunoprecipitates were analyzed by autoradiography. RG, radiograph; WB, Western blot; IP, immunoprecipitation; p-, phosphorylated.

Secondary organs (for review, see Refs. 12–16). Support for this notion can be found in the observation that tumor cell lines that do not express Fn such as MCF-7 cells exhibit poor tumorigenicity, growth, and angiogenesis (54), whereas overexpression of c-Jun in MCF-7 cells stimulated Fn synthesis and, concomitantly, increased tumorigenicity and invasiveness (55, 56).

In recent years our laboratory has been interested in the role of Fn in breast cancer lung metastasis. This interest was triggered by the fact that a survey of all our highly lung-metastatic breast cancer cell lines, including MTF7, MTF7L, MDA-MB-231, and 4T1 cells, revealed high levels of Fn expression (17) and by the observation that tumor cells isolated from the blood of tumor-bearing animals exhibited a characteristic cell surface coat of multiglobular polyFn. This phenotype could be reproduced in vitro, when cancer cells were subjected to EoE suspension culture in the presence of serum (9, 17). As indicated in the introduction, this unique surface expression of Fn was responsible for the docking of MT7 to the lung endothelial address in DPP IV (9, 17–21) and for facilitating lung colonization (9, 17, 20, 21). Interference with the polyFn/DPP IV adhesion by monoclonal antibodies (9), peptides directed against the DPP IV binding domains in the 13th, 14th, and 15th type III repeats of Fn (9, 17, 20), and a polypeptide representing the extracellular domain of DPP IV (20) abrogated metastatic colonization of the lungs. Here, we have presented some of the molecular underpinnings that regulate polyFn assembly in a rat model of hematogenous dissemination of breast cancer cells. We show that polyFn assembly critically depends upon the expression of endogenous Fn (Fn1) and is regulated by PKCe. This is evidenced by a significantly decreased polyFn assembly in cells transfected with either siRNA-PKCe or dominant-negative RD-PKCe and in cells treated with select PKC inhibitors. During a 1-h polyFn assembly phase, PKCe translocates in increas-

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The role of PKCe in polyFn assembly appears to be dualistic in that it may facilitate surface expression of endogenous, cellular Fn by promoting Fn exocytosis (62–66) and protein synthesis (67). In accordance, inhibitors of protein synthesis (e.g. cycloheximide) and exocytosis (e.g. monensin and brefeldin B) strongly impeded polyFn assembly (Fig. 2), albeit cells were kept in suspension culture in the presence of high concentrations of pFn. However, at this writing we have not identified any of the PKCe partner proteins that are required in regulating these processes. Nonetheless, inhibition of PKCe has been shown to trap integrin β1, a possible receptor for cellular Fn, in a CD81-positive intracellular compartment, and electron microscopy demonstrated the co-localization of PKCe and integrin β1 on vesicular membranes (68). Translocation of the integrin β1 from cytosolic vesicles to the plasma membrane appears to involve the PKCe-mediated phosphorylation of the cytoplasmic tail of β1-integrin at threonine 788/789 (69) or cytoskeletal intermediaries such as vimentin (70) and actin (62). Accordingly, interaction of PKCe with actin has been linked to the formation of invadopodia-like structures, increased pericellular metalloproteinase activity, and ultimately, invasiveness and metastasis in PKCe-transformed NIH3T3 fibroblasts (71). Whether the concomitant down-regulation of RhoC in MDA-MB-231 cells, in which PKCe expression was knocked down by RNA interference resulting in a cell phenotype that was significantly less proliferative, invasive, and metastatic (45), is intertwined in PKCe-actin, remodeling is unclear and needs further investigation. Supporting evidence for such a role may be deduced from the thrombin-induced PKCe-Rho-actin complex formation in actin reorganization in myofibroblasts (72). However, in our system of suspended (blood-borne) breast cancer cells, the Rho activity is significantly suppressed, and expression of constitutively active Rho suppresses polyFn assembly.4

In conclusion, our studies suggest that surface expression of polyFn occurs in a PKCe-dependent manner presumably involving the transport of Fn-receptor complexes from cytosolic vesicles to the plasma membrane and that such complexes serve as scaffolds for the subsequent augmentation of polyFn aggregates on tumor cells during their journey in Fn-rich blood plasma. This notion is supported by a dramatic inhibition of polyFn assembly by inhibitors of exocytosis and by siRNA knockdown of PKCe, which occurs even when cancer cells are exposed to high exogenous Fn concentrations and express several integrin and syndecan Fn receptors on their surfaces.3 Thus, we have disclosed here a novel regulatory principle for the recognized pro-metastatic role of Fn that may spur the design of new anti-metastatic therapies.
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