Epidermal Growth Factor Induces Fibroblast Contractility and Motility via a Protein Kinase C δ-dependent Pathway*

Received for publication, October 31, 2003, and in revised form, January 26, 2004 Published, JBC Papers in Press, January 27, 2004, DOI 10.1074/jbc.M311981200

Akihiro Iwabu, Kirsty Smith, Fred D. Allen, Douglas A. Lauffenburger, and Alan Wells

From the §Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, the §Biological Engineering Division, Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the *School of Biomedical Engineering, Drexel University, Philadelphia, Pennsylvania 19104

Myosin-based cell contractile force is considered to be a critical process in cell motility. However, for epidermal growth factor (EGF)-induced fibroblast migration, molecular links between EGF receptor (EGFR) activation and force generation have not been clarified. Herein, we demonstrate that EGF stimulation increases myosin light chain (MLC) phosphorylation, a marker for contractile force, concomitant with protein kinase C (PKC) activity in mouse fibroblasts expressing human EGFR constructs. Interestingly, PKCδ is the most strongly phosphorylated isoform, and the preferential PKCδ inhibitor rottlerin largely prevented EGF-induced phosphorylation of PKC substrates and MARCKS. The pathway through which EGF activates PKCδ is suggested by the fact that the MEK-1 inhibitor U0126 and the phosphatidylinositol 3-kinase inhibitor LY294002 had no effect on PKCδ activation, whereas lack of PLCγ signaling resulted in delayed PKCδ activation. EGF-enhanced MLC phosphorylation was prevented by a specific MLC kinase inhibitor ML-7 and the PKC inhibitors chelerythrine chloride and rottlerin. Further indicating that PKCδ is required, a dominant-negative PKCδ construct or RNAi-mediated PKCδ depletion also prevented MLC phosphorylation. In the absence of PLC signaling, MLC phosphorylation and cell force generation were delayed similarly to PKCδ activation. All of the interventions that blocked PKCδ activation or MLC phosphorylation abrogated EGF-induced cell contractile force generation and motility. Our results suggest that PKCδ activation is responsible for a major part of EGF-induced fibroblast contractile force generation. Hence, we identify here a new pathway helping to govern cell motility, with PLC signaling playing a role in activation of PKCδ to promote the acute phase of EGF-induced MLC activation.

Cell motility induced by activation of epidermal growth factor receptor (EGFR), and related receptor tyrosine kinases,

*These studies were supported by grants from the National Institute for General Medical Sciences and National Cancer Institute at the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence should be addressed: Dept. of Pathology, S17 21st Hall, University of Pittsburgh, Pittsburgh, PA 15261. Tel.: 412-647-7813; Fax: 412-647-8567; E-mail: wells@sx.msp.upmc.edu.

The abbreviations used are: EGFR, epidermal growth factor receptor; AP, alkaline phosphatase; CMV, cytomegalovirus; DAG, diacylglycerol; PKC, protein kinase C; PKD1, S-phosphoinositide-dependent protein kinase-1; PKC, protein kinase C; PLC, phospholipase C; PI, phosphatidylinositol; PI3K, PI 3-kinase; WT, wild type.

can be deconstructed into a series of orchestrated events: lamellipodial extension, formation of forward adhesions, exertion of contractile forces to pull the cell body forward, and detachment of the rear (1). While each process is required for net cell locomotion, it is not necessarily the case that signals downstream of receptor activation must concomitantly be involved in triggering all of the processes. Despite longstanding anecdotals, only recently have formal demonstrations emerged that signaling via EGFR actually elicits cell contractile force generation (2, 3) along with the other biophysical processes (4–6). Because of the central importance of growth factor-induced cell motility in physiological and pathological applications, such as organogenesis, wound repair, and tumor invasion, determination of key pathways involved in connecting EGFR activity to contractile force generation, as well as the other processes underlying motility, is a crucial undertaking.

Myosin motors operating on cytoskeletal actin filaments are presumed to be involved in growth factor-induced cell motility in manner similar to the roles they play in integrin-mediated migration (7). Myosin II is a prominent actor in this context. Myosin II is localized along with actin fibers in the protrusive anterior region and at posterior regions of motile cells, where it is thought to generate contractility, in organizing and breaking cell-substratum adhesion, and/or in reorganizing the actin cytoskeleton (8, 9). A recent report finds EGF to induce myosin II heavy chain phosphorylation (at least indirectly), with implications for subcellular localization of the active motor and consequent chemotactic cell movement (10). Phosphorylation at a regulatory serine 19 of the 20-kDa myosin light chain (MLC) subunit of myosin II promotes cell contractility in a variety of cell types responding to diverse stimuli. In addition, serine 19-phosphorylated MLC is enriched near membrane protrusion and retraction areas in motile fibroblast (11). The extent of MLC phosphorylation is regulated not only by protein kinases, such as Ca2+/calmodulin-dependent MLC kinase (MLCK) and Ca2+/calmodulin-independent Rho-kinase, but also by myosin phosphatase (MLCP) (12–14). During haptotactic migration, mitogen-activated protein (MAP) kinase ERK has been shown to mediate MLC phosphorylation through MLCK (15). Thus, the final myosin II contractile force generation machinery is engaged during both chemotactic and haptotactic motility. However, key signaling pathways through which EGFR activates myosin-based contractility have yet to be identified.

nase C substrate; MEK, mitogen-activated protein kinase kinase; MEM, minimum essential medium; MLC, myosin light chain; MLCK, myosin light chain kinase; MMTVp, mouse mammary tumor virus promoter; PKD1, S-phosphoinositide-dependent protein kinase-1; PKC, protein kinase C; PLC, phospholipase C; PI, phosphatidylinositol; PI3K, PI 3-kinase; WT, wild type.
Possible links between EGFR and myosin-based contractility are suggested by a few prior reports. Although ERK activity is required for MLC phosphorylation in haptotactic cell migration (15), ERK activity promotes the extension of membrane protrusions rather than retraction (16). A fair inference from these findings is that transcellular contractility is not solely driven by an ERK “master switch.” EGF-induced myosin heavy chain phosphorylation and localization requires a protein kinase C (PKC) intermediary (17). The PKC family of molecules is an attractive candidate for connecting EGF-elicited signals to myosin-mediated force generation, which is implicated in the contraction of muscle and non-muscle cells (18). PKC consists of a family of at least 11 isoforms. Specific isoforms of PKC are activated by phospholipids, diacylglycerol (DAG) generated by phospholipase C (PLC) or phospholipase D (PLD) from phosphatidylinositol 4,5-bisphosphate (PIP2), fatty acids generated by PLA2, and calcium, depending on isoforms. Based on their structural and biochemical properties these PKC isoforms can be divided into three major groups: (i) the classical PKC (cPKC; α, βI, βII, and γ), which are activated by DAG and are Ca2+-dependent; (ii) the novel PKC (nPKC; δ, ε, η, θ, and μ), which are activated by DAG but Ca2+-independent, and (iii) the atypical PKC (aPKC; ζ and λ), which do not respond to either DAG or calcium. Importantly, EGFR triggers PKC activity (19) at least in part downstream of phospholipase signaling (20). These data support the hypothesis that one or more PKC isoform, in the classical or novel groups, contributes to EGFR-mediated cell contractility during motility.

A picture of how PKC isoforms are regulated, in addition to final activation by lipid-containing molecules, has recently emerged in which direct phosphorylations play a major role (21, 22). cPKC and nPKC isoforms contain three conserved serine/threonine phosphorylation motifs of serine or threonine residues in the catalytic domain; a threonine in the activation loop (Thr-505 in PKCδ) and serines in the hydrophobic (Ser-643 in PKCδ and C-terminal (Ser-662 in PKCδ) regions (21). An upstream kinase, 3-phosphohistidine-dependent protein kinase-1 (PDK1), phosphorylates the activation loop (23, 24), which is necessary for the catalytic activity of cPKC isoforms (25, 26). The turn motif and hydrophobic sites then undergo autophosphorylation. The regulation of these sites in nPKC isoforms may mirror those of cPKC isoforms (27), though the existence of a heterologous upstream kinase has been inferred (28). These sequential phosphorylations render cPKC isoforms stable and ready for activation by DAG (29). However, PKCδ differs from cPKC in its regulation by phosphorylation (22). A study suggests that phosphorylation of the activation loop Thr-505 is not essential for subsequent activation; the PKCδ-specific acidic Glu-500 may assume at least some of the role of threonine phosphorylation (30). On the other hand, phosphorylation at Thr-505 by PDK1 is thought to be required for the stability of the enzyme (24). The functional consequence of phosphorylation of other Ser is also not settled, Li et al. (31) demonstrated that the mutation of Ser-643 markedly decreases PKCδ activity but Stempka et al. (30) showed that the same mutation has no effect on PKCδ catalytic function. Interestingly, the upstream PDK1 appears itself dependent on phospholipids, PIP3 in particular, for activation (32). In the present model, PI 3-kinase generates PIP3 after activation by receptors with tyrosine kinase, such as EGFR. These investigations, even with the uncertainties, point to many PKC isoforms as potential downstream effectors of growth factor receptor signaling.

In this present contribution, we show that EGFR signaling leads to MLC phosphorylation at serine 19 and consequently to cell contractile force generation and motility in fibroblasts. The pathway to myosin activation required activity of the novel PKCδ isoform. Moreover, we find that there appear to be two distinct phases of PKCδ activation, with PLCγ signaling required only for the acute phase. We have thus identified a novel pathway connection downstream of EGFR activation to one of the crucial biophysical processes underlying cell migration.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Rottlerin, selective PKCδ inhibitor (33), Go 6976, PKCδ/both selective inhibitor, U73122, PLC inhibitor, U73343, inactive analogue of U73122, LY294002, PI 3-kinase inhibitor, and ML-7, MLCK inhibitor were obtained from Biomol (Plymouth Meeting, PA). PD153035, EGFR tyrosine kinase inhibitor, U0126, mitogen-activated protein kinase kinase-1 (MEK-1) inhibitor, and chelerythrine chloride, pan-PKC inhibitor were obtained from Calbiochem (La Jolla, CA). Human recombinant EGF was obtained from BD Biosciences (San Jose, CA). Type I collagen was from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against phosphorylated PKC (pan), phosphorylated (Ser) PKC substrate, phosphorylated MARCKS (myristoylated alanine-rich protein C kinase substrate) (Ser-152/156), phosphorylated PKCδ (Ser-643), phospho-ERK (p44/42), phospho-Akt (Ser-473, 587F11), and phosphorylated MLC (Ser-19) were obtained from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal antibody against MLC (FL-172), nPKCδ (C-20), and cPKCα (C-20) were from Santa Cruz Biotechnology. Type I collagen was from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal antibodies against phosphorylated PKC (pan), phosphorylated (Ser) PKC substrate, phosphorylated MARCKS (myristoylated alanine-rich protein C kinase substrate) (Ser-152/156), phosphorylated PKCδ (Ser-643), phospho-ERK (p44/42), phospho-Akt (Ser-473, 587F11), and phosphorylated MLC (Ser-19) were obtained from Cell Signaling Technology (Beverly, MA). The rabbit polyclonal antibody against MLC (FL-172), nPKCδ (C-20), and cPKCα (C-20) were from Santa Cruz Biotechnology. The rabbit polyclonal antibody against α-actin was from Sigma-Aldrich. All cell culture reagents...
EGF Induces MLC Phosphorylation via PKCδ

were obtained from CellGro (Herndon, VA) or Invitrogen.

Cell Culture—The establishment and maintenance of the NB6 WT, expressing full-length wild-type EGF, or NB6 c973, expressing signaling-restricted EGF lacking all autophosphorylation motifs and fails to activate PLCγ, cell lines have been described (19, 34, 35). Briefly, these constructs were retrovirally transduced in NB6 cells, Swiss 3T3-derived fibroblasts, which lack endogenous EGFRs (35). Cells were cultured in a minimum essential medium (MEM)–α with 7.5% fetal calf serum plus 2 mM l-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM, nonessential amino acids, and the antibiotics penicillin (100 units/ml), streptomycin (100 μg/ml), and G418 (350 μg/ml) as the growth medium. Subconfluent cells were passaged with a 1:8 split ratio at 3-day intervals using 0.25% trypsin with 0.05 mM EDTA. Cells were questioned using restricted or no serum conditions without G418 prior to experiments.

Plasmid Construction and Transfection—The dominant-negative (DN) PKCδ construct (36) was a generous gift from Dr. Michael Simons (Dartmouth Medical School). The CN construct was generated by replacing the conserved lysine 376 in the ATP binding domain with tryptophan. For stable expression, DN PKCδ DNA was subcloned into pCEP4 (Invitrogen) hygromycin resistant vector downstream from a cytomegalovirus promoter (CMVp). CMVp was replaced with a mouse mammary tumor virus promoter (MMPW) for inducible expression (20). The empty pCEP4 vector, CMVp of which was replaced with MMTV, was used as control. The construct was stably transfected into NB6 WT cells by electroporation. Cells were trypsinized, pelleted, and resuspended in Opti-MEM medium (Invitrogen) in electroporation cuvette, and the plasmid was added to a total of 30 μg. The cells were electroporated at 0.3 kV and 950 μF (Gene Pulser, Bio-Rad). 48 h after electroporation, cells were selected in the growth medium containing hygromycin B (Calbiochem) (100 μg/ml). Polyclonal cell lines consisting of more than 20 colonies were established. Two independent electroporations and stably transfected cell lines were established and tested. Dexamethasone (2 μM for 24 h) was used to induce MMTV-driven DN PKCδ expression at the same time with quiescence.

siRNA Transfections—siRNA duplexes (siRNAs) were synthesized and purified by IDT (Coralville, IA). The siRNA sequence for targeting PKCα (GenBank™ accession number NM_011103) was PKCα siRNA (5′-AGAUCUUCCGAAAAGGACCGTT-3′). The siRNA sequence for targeting PKCδ (GenBank™ accession number NM_011101) was PKCδ siRNA (5′-ACAACUGGAGACAGUGAATT-3′). GFPsiRNA (5′-GAC-GUGCGGCGCGGUGAAGTT-3′) was used as a negative control (37). Transfection of siRNAs was performed using the manufacturer’s protocol for LipofectAMINE™ 2000 (Invitrogen). Briefly, 4 μl of 20 μM siRNA was mixed with 200 μl of Opti-MEM. 4 μl of LipofectAMINE™ 2000 was diluted in 200 μl of Opti-MEM and incubated at room temperature for 5 min. After the incubation, the diluted LipofectAMINE™ 2000 was combined with the diluted siRNA and then incubated for an additional 20 min at room temperature. Total 400 μl of siRNA-LipofectAMINE™ 2000 complexes was applied to each well of cultured NB6 WT fibroblasts at ~70% confluence in a 6-well plate.

Immunoblotting and Immunoprecipitation—Cells were grown to confluence in 6-well tissue culture plastic plates. After 24 h of quiescence in MEMα with 0.5% dialyzed fetal calf serum, cells were treated. Cells were lysed with sodium dodecyl sulfate (SDS)-sample buffer containing 0.1 M Tris-HCl, 4% SDS, 0.2% b-mercaptoethanol. Cell lysates were separated by SDS-PAGE and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore, Bedford, MA). Blots were probed by primary antibodies before visualizing with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) followed by visualization with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated antibodies (Promega, Madison, WI). Blots were probed by primary antibodies before visualizing with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) followed by visualization with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated antibodies (Promega, Madison, WI). Blots were probed by primary antibodies before visualizing with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI) followed by visualization with alkaline phosphatase-conjugated or horseradish peroxidase-conjugated antibodies (Promega, Madison, WI).

For immunoprecipitation, cells were prepared and treated as described above. Cells were lysed with radioimmune precipitation lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, and 0.5% deoxycholate) containing 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were cleared by centrifugation at 13,000 × g for 15 min. Soluble proteins were incubated with anti-PKCδ antibody for 4–6 h at 4 °C. Immunocomplexes were incubated with protein G-agarose and centrifuged. The pellets were washed three times with radiolabeled immunoprecipitation buffer containing protease inhibitors. Precipitated proteins were subjected to analyze by immunoblotting as described above.

Motility Assay—EGF-induced migration was assessed by the ability of the cells to move into an acellular area (38). Cells were plated on 6-well plastic dishes and grown to confluence in MEM-α with 7.5% fetal bovine serum. After 24 h quiescence, an area was demarcated by a rubber policeman. Cells were then treated with or without EGF (10 ng/ml) and incubated at 37 °C; inhibitors or diluent alone were added at the same time as EGF. Photographs were taken at 0 and 24 h, and the relative distance traveled by the cells was determined.

Isometric Force Measurement—The preparation of three-dimensional fibroblast populated collagen lattices (FPCL) has been described previously (2). Briefly, FPCL containing 7.5 × 10^5 cells were prepared with modifications of Kolodney and Elson (39). A mixture of cells and 0.75 mg/ml type I collagen was poured into the annular space of cylindrical Teflon molds (1 ml/mold) and gelled at 37 °C in 5% CO2 for 1 h. Ring-shaped FPCL gels were removed from the molds and placed in tissue culture medium for ~40 h, maintaining the original diameter (15 mm) of the FPCL with sterile parallel spacing rods. This configuration established a tensed FPCL at the time of force measurement. Contractile force measurements of the WT NB6 and c973 fibroblasts were performed with an isometric force transduction system. The system uses force transducers (model 52-9545, Harvard Apparatus, South Natick, MA) and mounts one FPCL per transducer vertically. The mounted tissue was placed in an organ bath containing 50 ml HEPES-

**Fig. 2.** EGF-induced compaction (A) and motility (B) are dependent on the MLCK. Inhibition of MLCK by ML-7 abrogates cell compaction and motility in the NB6 WT cell line. Cell motility and compaction assays were performed as described under “Experimental Procedures.” MLCK selective inhibitor ML-7 (20 μM) was added at the same time as EGF (1 μg/ml). A, gel compaction values are shown as the percentage of nontreated gels. The data in the graph are the mean ± S.E. of three independent experiments, with each experiment was performed in triplicate. Statistical analysis was performed by Student’s t test: *, p < 0.05; **, p < 0.01. B, cell motility was calculated as fold increase over basal traveling distance of nontreated cells. The data in the graph are the mean ± S.E. of three independent experiments, with each experiment was performed in triplicate. Statistical analysis was performed by Student’s t test: **, p < 0.01.

Downloaded from http://www.jbc.org/ on July 25, 2018
EGF Induces MLC Phosphorylation via PKC

A

EGF (nM) for 30 min. Cells were then lysed, and equal volumes of were treated or not with increasing concentrations of EGF (0.01, 0.1, 1, and quiesced for 24 h. Cells were harvested from monolayer culture using 0.25% trypsin/EDTA and resuspended in quiescent media (serum-free medium containing 1 mg/ml bovine serum albumin). Cells were incubated with inhibitors for 30 min prior to matrix preparation. Neutralized collagen solutions (1 mg/ml containing 10^5 cells/ml, ± EGF (1 μg/ml) and inhibitors (or diluent alone) were dispensed into 24-well culture plates (0.5 ml solution/well). Collagen solutions were left to polymerize for 60 min at 37 °C in a humidified incubator with 5% CO_2 and then each matrix was overlaid with 1 ml of quiescent medium ± EGF (1 μg/ml) and inhibitors. The matrices were gently released from the surface and sides of each well using a scalpel and incubated for 24 h. Compaction was determined by weighing the matrices after the incubation period. Data are shown as the percentage of the control matrix weight.

Cell viability was assessed following compaction by removal using collagenase digestion. Matrices were washed in phosphate-buffered saline and then incubated with 0.05% trypsin for 10 min followed by collagenase (0.25 mg/ml) for a further 10 min. Fetal bovine serum (20%) was added to quench the collagenase activity and cell viability was determined by trypan blue staining. Cell viability was not significantly affected (<10%) by ML-7 at 20 nm.

**Statistical Analyses**—All data are expressed as means ± S.E. of separate experiments. Differences between means were determined by Student’s t test for unpaired samples, and those at p < 0.05 were considered significant.

**RESULTS**

**EGF Induces Phosphorylation of Myosin Light Chain—**Exposure of fibroblasts to EGF leads to cell contractility and motility (2, 38), both of which are proposed to require actomyosin-based contraction (1). To verify this, we found that EGF leads to phosphorylation of MLC at the activation-specific site serine 19 (Fig. 1). This occurred rapidly in cells expressing the wild type EGFR, but dissipated by more than half over the ensuing 2 h (see below). As expected, EGF-induced phosphorylation was blocked by the EGFR-selective inhibitor PD153035 and the MLCK-selective inhibitor ML-7. Similarly, EGFR-mediated compaction and motility was blunted by ML-7 (Fig. 2). While these data were anticipated, they establish directly for the first time that EGF leads to MLC phosphorylation and that motility depends on this contractile mechanism.

**EGF Induces Activation of PKC with PKCα as a Major Isoform Activated—**To determine the key pathway(s) from EGFR to MLC, we focused on PKC. EGF has been shown to induce phosphorylation of MLC, which leads to phosphorylation of MLC at the activation-specific site (41). PKC leads to phosphorylation of MLC, which leads to the activation of PKC targets MARCKS and other targets (39). While these data were anticipated, they establish directly for the first time that EGF leads to MLC phosphorylation and that motility depends on this contractile mechanism.

FIG. 3. PKC activity in NR6 cells was determined by confluence and quiesced for 24 h. A, PKC is activated in response to EGF. Cells were treated or not with increasing concentrations of EGF (0.01, 0.1, 1, and 10 nM) for 30 min. Cells were then lysed, and equal volumes of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-phospho-PKC(pan) and anti-phospho-MARCKS anti-phospho-(Ser) PKC substrates and anti-α-actin. B, pan-PKC inhibitor chelerythrine chloride blocks EGF-induced PKC activation. Quiescent cells were either left untreated or preincubated with increasing concentrations of chelerythrine chloride (5, 10, and 100 nM) for 15 min before challenge with 10 nM EGF for 30 min. Nontreated cells were used as control (Con). Cells were then lysed, and cell lysates were analyzed for phospho-PKC(pan), phospho-MARCKS, phospho-(Ser) PKC substrates, and α-actin by immunoblotting. C, EGF-induced PKC activation is downstream of EGFR. Quiescent cells were treated in the absence or presence of 10 nM EGF for 30 min with or without pretreatment of PD153035 (1 μM) for 15 min. Cells were then lysed, and cell lysates were analyzed for phospho-PKC(pan), phospho-MARCKS, phospho-(Ser) PKC substrates, and α-actin by immunoblotting. Shown are representative blots of at least three repeats at all data points.

buffered, serum-free Dulbecco’s MEM (37 °C). Each FPCL was permitted to reach an equilibrium force over a period of 30 to 60 min prior to addition of 10 nM EGF. The contraction force response of the FPCL was monitored by computer data acquisition. The strain level of the FPCL was maintained throughout so as to measure both the initial contractile force and the persistence of contractile force as a function of time.

**Gel Compaction Assay—**NR6 WT fibroblasts were grown in floating collagen type I matrices using a modification of described methods (40). Collagen solutions were left to polymerize for 60 min at 37 °C in a humidified incubator with 5% CO_2 and then each matrix was overlaid with 1 ml of quiescent medium ± EGF (1 μg/ml) and inhibitors. The matrices were gently released from the surface and sides of each well using a scalpel and incubated for 24 h. Compaction was determined by weighing the matrices after the incubation period. Data are shown as the percentage of the control matrix weight.
immunoblotted with anti-phospho-PKC. Nontreated cells were used as control (Fig. 5). Cells were then lysed, and equal volumes of proteins were separated by 10% SDS-PAGE and immunoblotting. Shown are representative blots of three independent experiments at all data points.

Calcium (42). Of interest, phosphorylation of PKC was noted when the WT EGFR-expressing cell lines, which fails to activate PLC. Quiesced cells were treated in the absence or presence of EGF (10 nM) for 30 min with or without pretreatment of 1 μM PD153035 for 15 min. Cells were then lysed, and cell lysates were analyzed for phospho-PKC and p-MARCKS phosphorylation. Shown are representative blots of three independent experiments at all data points. D, rottlerin inhibits EGF-induced PKC activation and PKC substrates phosphorylation. NR6 WT cells were grown to confluence. After quiescence, cells were pretreated or not with chelerythrine chloride (10 μM) for 15 min before challenge with 10 nM of EGF for 30 min. Nontreated cells were used as control (Con). Cells were then lysed, and equal volumes of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-phospho-PKC, anti-phospho-MARCKS, anti-phospho-(Ser) PKC substrates, and anti-α-actin. Shown are representative blots of at least three independent experiments at all data points.

**Fig. 4. PKCδ comprises the majority of EGF-induced PKC activity.** A. phosphorylation of PKCa/βII, though present, is not enhanced by EGF treatment. NR6 WT cells were grown to confluence. After quiescence, cells were pretreated or not with chelerythrine chloride (10 μM) for 15 min prior to EGF treatment (10 nM, 30 min) and lysed. Same volumes of cell lysates were size-fractionated with anti-phospho-PKCa/βII and anti-α-actin. B, quiesced cells were treated in the absence or presence of EGF (0.01, 0.1, 1, and 10 nM) for 30 min prior to immunoprecipitation of PKCδ. Precipitated proteins were analyzed for phospho-PKCδ and total PKCδ by immunoblotting. Immunoblots for phospho-PKCδ on total cell lysates presented the same results (data not shown). C, PKCδ phosphorylation is enhanced by EGF in both NR6 WT and NR6 c973 cell lines, which fails to activate PLC. Quiesced cells were treated in the absence or presence of EGF (10 nM) for 30 min with or without pretreatment of 1 μM PD153035 for 15 min. Cells were then lysed, and cell lysates were analyzed for phospho-PKCδ and α-actin by immunoblotting. Shown are representative blots of three independent experiments at all data points. D, rottlerin inhibits EGF-induced PKCδ activation and PKC substrates phosphorylation. NR6 WT cells were grown to confluence and quiesced for 24 h. Quiesced cells were either left untreated or preincubated with increasing concentrations of rottlerin (1, 3, and 10 μM, 30 min) for 15 min before challenge with 10 nM of EGF for 30 min. Nontreated cells were used as control (Con). Cells were then lysed, and equal volumes of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-phospho-PKC, anti-phospho-MARCKS, anti-phospho-(Ser) PKC substrates, and anti-α-actin. Shown are representative blots of at least three independent experiments at all data points.

The major contributor to phosphorylation of PKC targets as the isoform-preferential inhibitor rottlerin blocked the majority of phosphorylation of MARCKS and other protein targets (Fig. 4), whereas the PKCa/βII inhibitor Gö 6976 had little effect (data not shown).

**PLCy but Neither ERK nor PI3K Contributes to PKCδ Activation**—Multiple PKC isoforms are activated downstream of the generation of diacylglycerol (DAG) and calcium fluxes (these secondary to IP3-mediated release). In particular, EGFR triggers PLCγ to generate both DAG and IP3 resulting in PKC activities (19, 20). Interestingly, EGFR activation in NR6 cells leads to only weak calcium fluxes (42, 43); this is consistent with the mode of activation of PKCδ regulation by allosteric lipids but independent of calcium. To probe this link between EGFR and PKCδ, we used both the PLC-specific inhibitor U73122 and NR6 c973 cells that express the truncated c973 EGFR, which fails to activate PLCγ, generate IP3, or mobilize calcium (42). Of interest, phosphorylation of PKCδ was delayed in the cells expressing c973 EGFR (Fig. 5). This is most probably due to the inability to activate PLCγ as a similar delayed phosphorylation was noted when the WT EGFR-expressing cells were challenged in the presence of the PLC inhibitor U73122 (Fig. 5). The inactive congener U73343 had no effect on phosphorylation of PKCδ or MLC (data not shown). This suggests that while PLCγ is not strictly required for PKCδ phosphorylation, it contributes to the early phase of activation, a situation not unprecedented (44).

The pathway to the later phase of PKCδ activation is unknown at present. However, it is unlikely to involve either of the well-described ERK or PI 3-kinase pathways (Fig. 6). ERK activation was completely abrogated by the MEK inhibitor U0126 without any affect on PKCδ phosphorylation. Similarly, even though EGF does not activate PI 3-kinase robustly in these fibroblasts, inhibitory concentrations of LY294002 had no impact on EGF-induced PKCδ phosphorylation.

**PKCδ Is Required for EGF-induced Phosphorylation of MLC**—Significance of the findings above is buttressed if PKC δ signaling leads to actomyosin effects. EGF-induced phosphorylation of myosin light chain was completely abrogated not only by the pan-PKC inhibitor chelerythrine, but the PKCδ isoform-preferential inhibitor rottlerin (Fig. 6). That this loss of MLC phosphorylation did not arise from pancellular inhibitory effects was shown by ERK phosphorylation being maintained even at the highest rottlerin concentrations. Of course, no single inhibitor is truly specific, and rottlerin also uncouples mitochondrial ATP production (45). Thus, an alternative mode of signal abrogation of the PKCδ isoform was required. Expression of a dominant-negative PKCδ construct (36) in the NR6 WT cells completely prevented phosphorylation of both PKCδ and MLC (Fig. 7). Similarly, PKCδ gene silencing with PKCδsiRNA, a more specific mode of inhibition, abolished EGF-induced MLC phosphorylation, while control transfections with GFPsiRNA or PKCasiRNA had no effect on this signaling pathway (Fig. 7).

If PKCδ is a critical signaling intermediary to MLC activation, then interventions should show similar effects for phosphorylation of MLC as they did for PKCδ (Fig. 6). Again, U0126 and LY294002 did not diminish EGF-induced phosphorylation of MLC (Fig. 6). Interestingly, use of the PLC inhibitor U73122 and stimulation of cells expressing the truncated c973 EGFR also displayed delayed phosphorylation of MLC (Fig. 8). These profiles are strikingly similar to the effects on PKCδ phosphorylation, further strengthening the upstream position of PKCδ in MLC activation.
PKCα Is Required for EGF-induced Cell Motility and Contractility—Contractility is considered necessary for motility to pull the cell body forward (1); this was confirmed in our system as ML-7 prevented this EGF-induced motility (Fig. 2). As such if PKCα is the key intermediary in this cascade, then its inhibition should also block motility. All approaches for inhibiting PKCα signaling pathway, the pharmacologic agent rottlerin, the molecular dominant-negative PKCδ, and PKCα siRNA, abrogated EGF-induced motility (Fig. 9). Interestingly, inhibition of PKCα signaling, decreased even basal motility. This is not unexpected, similarly to the decrement in basal motility noted in the face of ML-7 (Fig. 2), as haptokinesis also requires PLC kinase activity (15). Still, that the PKCα siRNA abrogation did not reduce basal motility, may suggest either a lesser decrement in signal reduction using this approach (as noted by some EGF-induced motility retained) or non-PKCα-specific effects of rottlerin and the DN PKCδ. Compaction of floating collagen gels was similarly blocked by rottlerin (Fig. 10). Both modes of disrupting this putative signaling cascade resulted in significantly reduced gel compaction.

We found a two-stage MLC phosphorylation effect above, with PLCγ contributing to acute signaling, but later signaling was PLC-independent. While the time-scale integration of motility would not allow us to discern this 30–60 min delay (46), we could parse this by isometric gel contraction (2). We found cells expressing the c973 EGFR that fails to activate PLCγ did induce robust contraction but that this response was delayed compared with cells expressing WT EGFR (Fig. 10). EGF induced peak contractility at 0.38 ± 0.10 h in cells expressing the WT receptor, compared with 1.98 ± 0.10 h in those expressing c973 EGFR (n = 5; p < 0.001); however, the peak force was similar between the cell types (25.1 ± 1.3 dynes in WT versus 24.1 ± 3.2 in c973 NR6 cells). Thus, the cell responses of motility and contraction correlate directly to at least two independent pathways leading from EGF to PKCα and then to myosin-based contraction.

DISCUSSION

Cell motility results from a highly orchestrated set of events triggered by external signals (1, 7). While the crucial cell processes underlying migration identified by deconstructive analyses, protrusion, de novo adhesion, contraction, and rear release, are all involved in productive motility, only experimental evidence can determine which operate as active governors and which as permissive functions for the various modes of motility induction. This distinction is critical to the modulation of motility, especially for therapeutic purposes, because active governors should proffer for a more targeted intervention. In this report, we tested the hypothesis that active PKC signaling is a key regulatory element in EGF-induced contractility during fibroblast motility. We provide evidence that EGF induces serine 19 phosphorylation of MLC and that this lies downstream of the novel PKC isoform, PKCδ. Molecular or pharmacologic inhibition of PKCδ blocks subsequent MLC phosphorylation, contractility and motility. Furthermore, we also demonstrate that PLCγ is responsible for a major portion, the acute phase, of the EGF-induced fibroblast contractility. These data provide for a novel target for intervention to regulate growth factor-induced motility.

Orchestration of cell migration requires that multiple biochemical pathways be coordinated. Certain end effectors for the crucial biophysical processes have been identified recently. The small GTPases of the Rho family and actin binding proteins control the actin cytoskeleton reorganization required for protrusion at the front (47–51). Some of these same molecular switches regulate the formation of new adhesions (52, 53). At the other end of the cell, the intracellular limited protease c973 EGFR (54–57), particularly on moderately to highly adhesive substrata (58). These end effectors appear common between motility signaled by adhesion receptors and that triggered by growth factor receptors. However, the signaling pathways that get to the end effectors appear distinct. EGF and other growth factor receptors require PLCγ signaling to promote cytoskeletal reorganization at the front (34, 48, 59, 60), while haptotaxis occurs in its absence (34, 61). Rear de-adhesion triggered by EGF, IGF-1, or PDGF is limited when ERK or m-calpain is inhibited (3, 55), while basal haptokinesis is limited by interference with the μ-calpain isof orm (55, 62). Further, during haptokinesis, ERK signaling has been linked to cell contactility, leading to MLCK activation and phosphorylation of serine19 on MLC (15). Herein, we report that inhibition of ERK blocks neither activation of PKCα nor phosphorylation of MLC. This is consistent with our earlier finding that ERK inhibition prevented fibroblast detachment and channeled the cell contractility from motility to compaction of the matrix (2). Rather, the same end effect of myosin II contraction is achieved via a PKCα-dependent pathway.

Association of particular signaling pathways with individual biophysical processes that are distinct from those for haptokinesis and diverge at the immediate postreceptor step carries implications for the regulation of motility and for targeted interventions. Divergence of signaling to separate biophysical processes of motility clearly provides for the possibility that these signals and processes can generate active locomotion under some circumstances but alternative behaviors under others. For instance contractility in the absence of rear de-
adhesion leads to matrix contraction (2), a cellular event required for dermal reorganization late in repair. Decoupling of active de-adhesion and active contractility may enable motility under conditions of low adhesiveness (58) relevant to migration into a provisional wound matrix or hyaluronic acid-filled space. Another speculation can center on contraction and de-adhesion in the absence of protrusion being operative to enable compromise of single cell layered endothelium and epithelium during

FIG. 6. MLC phosphorylation occurs downstream of PKCδ, but not ERK or PI 3-kinase. NR6 WT cells were grown to confluence and quiesced for 24 h. A and C, treating the cells with chelerythrine chloride or rottlerin completely inhibited EGF-induced MLC phosphorylation. Quiesced cells were either left untreated or preincubated with chelerythrine chloride (10 μM) for 15 min (A) or rottlerin (1, 3, and 10 μM) for 30 min (C) before stimulation in the absence or presence of 10 nM EGF for 30 min. Nontreated cells were used as control (Con). Cells were then lysed, and cell lysates were analyzed for phospho-MLC, MLC (total), and phospho-ERK (p44/42, Cell Signaling Technology), by immunoblotting. B, and D, ERK and PI 3-kinase do not lead to EGF-induced PKCδ activation or EGF-induced MLC activation. Cells were either left untreated or pretreated with U0126 (5, 10, 20, and 50 μM) (B) or LY294002 (10 μM) (C) for 30 min. Nontreated cells were used as control (Con). The cells were then stimulated with EGF (10 nM) for 30 min and lysed. Cell lysates were analyzed for phospho-PKCδ, phospho-MLC, phospho-ERK, phospho-Akt, and α-actin, by immunoblotting. Shown are representative blots of at least two repeats at all data points.

FIG. 7. PKCd mediates EGF-induced MLC phosphorylation. A, DN PKCd-containing (NR6 WT-DN PKCd) and control empty vector-containing (NR6 WT-Mock) NR6 WT cells were tested for phosphorylation of downstream targets. Cells were quiesced with 2 μM dexamethasone for 24 h prior to treatment or not with EGF (10 nM) for 30 min. Cell lysates were immunoblotted with anti-phospho-PKCδ and anti-phospho-MLC followed by stripping and then reprobed with anti-α-actin and anti-MLC (total), respectively. B and C, RNA interference with siRNA transfection was performed as described under “Experimental Procedures.” 24 h after transfection, cells were quiesced for 24 h. B, cells were lysed, and equal volumes of proteins were separated by 10% SDS-PAGE and immunoblotted with anti-PKCδ (total), anti-PKCα, and anti-α-actin. C, quiesced cells were treated or not with EGF (10 nM) for 30 min. Cells were then lysed, and cell lysates were analyzed for phospho-PKCδ, phospho-MLC, and α-actin by immunoblotting. Shown are representative blots of at least two repeats at all data points.
infection. Separating the key switches between haptokinesis and chemokinesis in response to growth factors suggests that these two modes of motility accomplish fundamentally distinct functions. While these concepts are speculative at present, the existence of process, and induction mode, specific molecular switches enables us to intervene specifically both in discovery and, potentially, in therapies aimed at angiogenesis and tumor invasion.

The definition of this new pathway from EGFR to cell contractility implicating PKC\(\alpha\)/H9254 appears at odds with an earlier report. In vitro, using purified enzymes and substrates, PKC was shown to phosphorylate MLC at serine 1, serine 2, and threonine 9, leading to inhibition of MLC function (63). Possible reasons for this discrepancy lie in the different approaches; we are examining in vivo, and likely indirect effects of PKC\(\alpha\)/H9254 on MLC whereas the earlier work was in vitro and direct. The recent discovery that PKC phosphorylates the CPI-17 phosphoprotein, a potent inhibitor of MLC phosphatase (64), provides both an explanation for this discrepancy and a potential pathway from PKC\(\alpha\)/H9254 to MLC phosphorylation. However, whether this connection is operative in our cells and in wound fibroblasts and is the only connection between PKC\(\alpha\) and myosin-based contractility, or whether there is a parallel analogous pathway as CPI-17 may not be present in fibroblasts (65), is actively under study but lies beyond the scope of the present communication.

**Fig. 8.** Lack of PLC signaling correlates with delayed phosphorylation of MLC. A, NR6 WT cells and NR6 c973 cells, which fails to activate PLC, were grown to confluence and quiesced for 24 h. Cells were treated with EGF (10 nM) in the absence or presence of 1 \(\mu\)M U73122, PLC inhibitor, for 0, 10, 30, 60, 120, and 240 min. Cells were then lysed, and equal volumes of proteins were separated by 15% SDS-PAGE and immunoblotted with anti-phospho-MLC followed by stripping and then reprobed with anti-MLC (total). Shown are representative blots of at least three repeats at all data points. B, phosphorylated MLC levels were enumerated by densitometry (NIH Image) and calculated as a ratio of total MLC. The data in the graph are the mean \pm S.E. of three independent experiments. Statistical analysis was performed by Student's t test: *, \(p < 0.05\); **, \(p < 0.01\).

**Fig. 9.** PKC\(\alpha\) inhibition abrogates EGF-induced cell motility. A, cell motility assay was performed with NR6 WT cells in the absence or presence of EGF (10 nM) in the absence or presence of rottlerin (3 \(\mu\)M) and Go 6976 (100 nM), respectively, as described. Cell motility was calculated as -fold increase over basal traveling distance of nontreated cells. B, dexamethasone-inducible DN PKC\(\alpha\)-transfected NR6 WT cells (NR6 WT-DN PKC\(\alpha\)) and the control vector-transfected NR6 WT cells (NR6 WT-Mock) were examined for EGF-induced motility assay. Cells were quiesced with or without 2 \(\mu\)M dexamethasone (Induction) for 24 h. Then, cell motility assay was performed in the presence or absence of EGF (10 nM) as described. Cell motility was calculated as -fold increase over basal traveling distance of nontreated and not DN PKC\(\alpha\) induced NR6 WT-DN PKC\(\alpha\) cells. The data in the graph are the mean \pm S.E. of at least two independent experiments, with each experiment was performed in triplicate. Statistical analysis was performed by Student's t test: **, \(p < 0.01\).
A second superficially conflicting aspect might be from earlier reports on PKC activation limiting motility. Strong activation of PKC by PMA has long been known to variously affect cell motility and invasive behavior (66–68). Conversely, inhibition of PKC has been shown to inhibit cell motility and invasiveness (69–71). In contrast, we link activation of PKC6 to increased contractility and subsequent motility; of specific interest is that PKC6 activity comprises the majority of detectable EGFR-triggered PKC activity in these cells; gene expression analyses of these cells detected appreciable levels of only PKCα and PKCδ (data not shown). However, again we propose that the seeming discrepancy is related to the nature and mode of signaling. In these earlier studies the PMA activation was supraphysiologic resulting in high level activation and subsequent down-regulation of classical and novel PKC isoforms. As PKC is able to phosphorylate numerous cytoskeletal and focal adhesion proteins, it is not surprising that pharmacologic and tonic activation of PKCs have pleiotropic effects on cell adhesion and thus motility. In fact, we find that pharmacologic levels of PMA and other phorbol esters abrogate all cell motility in NR6 fibroblasts (data not shown). Herein, we activated PKCδ via a physiological mechanism subject to feedback attenuation (19, 20). Thus, the PKC activity is likely more regulated in a physiologically relevant manner.

It may not necessarily surprise that the operative PKC isoform is the novel PKCδ. While at least the acute phase of PKC activity is downstream of PLCγ signaling, PLC signaling is required for the total activation of PKC as demonstrated in the presence of the pan-PLC inhibitor U73122 or downstream of c973 EGFR, which fails to activate PLC (34). Furthermore, even though EGFR strongly activates PLCγ, calcium mobilization is weak in the NR6 cells (42). More recently, we have found that an EGF-induced calcium flux can be detected in only 20% of the cells exposed (43), while phospho-PLCγ is found and IP3 is generated in practically all cells (47, 48). Thus, a calcium-independent isoform is more likely to be activated in these cells downstream of PLCγ. Intriguing, the EGF-induced PKCδ phosphorylation at Ser-643 was not inhibited by the PI3K inhibitor LY294002, although PI3K regulates PDK1. Le Good et al. (23) reported that LY294002 blocked serum-stimulated PKCδ phosphorylation at Thr-505, with Ser-643 phosphorylation as subsequent autophosphorylation (22). However recently, Sonnenburg et al. (72) have shown that PDK1 may phosphorylate cPKC by a PI3K-independent mechanism (72). This other pathway might also control EGF-induced PKCδ phosphorylation in our cells; as the multiple stimulatory molecules in serum are distinct from EGFR ligands, discrepancies in upstream signaling pathways between serum and EGF are not unexpected (6). Regardless, the mechanism by which the late phase of PKCδ activation occurs remains to be determined in subsequent studies.

In summary, our results altogether indicate that PKCδ-mediated MLC phosphorylation, presumably through MLCK, plays a pivotal role in growth factor-induced motility by promoting cell motility. Our findings now open further questions such as what is the pathway between PKCδ and MLCK activation, and how EGF activates PKCδ in the late phase. Therefore, further investigations are needed to clarify this connection.

**Acknowledgments**—We thank Dr. Michael Simons (Dartmouth Medical School) for the dominant-negative PKCδ construct. We thank the members of the Wells and Lauffenburger laboratories and Drs. Cary Wu and Frank Gertler for critical insights and discussions.

**REFERENCES**

1. Lauffenburger, D. A., and Horwitz, A. F. (1996) Cell 84, 359–369
2. Allen, F. D., Asnes, C. F., Chang, P., Elson, E. L., Lauffenburger, D. A., and Wells, A. (2002) Wound Repair Regen. 10, 67–76
3. Haase, I., Evans, R., Pofahl, R., and Watt, F. M. (2003) J. Cell Sci. 116, 3227–3238
4. Gladig, A., Lauffenburger, D. A., and Wells, A. (2002) Trends Cell Biol. 12, 46–54
5. Ware, M. F., Wells, A., and Lauffenburger, D. A. (1998) J. Cell Sci. 111, 2423–2422
6. Wells, A., Gupta, K., Chang, P., Swindle, S., Glading, A., and Shiraha, H. (1998) Microsc. Res. Tech. 43, 395–411
7. Mitchison, T. J., and Cramer, L. P. (1996) Cell 84, 371–379
8. Conrad, A., Jaffredo, T., and Conrad, G. (1996) Cell Motil. Cytoskeleton 31, 93–112
9. Gea, A., and Taylor, D. L. (1993) J. Cell Biol. 121, 1095–1107
10. Bresnick, A. (1999) Curr. Opin. Cell Biol. 11, 26–33
11. Matsumura, F., Ono, S., Yamakita, Y., Totsukawa, G., and Yamashiro, S. (1998) J. Cell Biol. 140, 119–129
12. Hartshorne, D., Bo, M., and Erdlida, F. (1998) J. Muscle Res. Cell Motil. 19, 325–341
13. Kamm, K., and Stull, J. (2001) J. Biol. Chem. 276, 4527–4530
14. Somlyo, A. P., and Somlyo, A. V. (2000) J. Physiol. 523, 177–185
15. Klemke, L. R., Cai, S., Giannini, A., Gallagher, P., Delanerolle, P., and Cheres, D. A. (1997) J. Cell Biol. 137, 481–492
16. Brahmabandh, A., and Klemke, R. L. (2003) J. Biol. Chem. 278, 13916–13925
17. Straussman, S., Even, L., and Ravid., S. (2001) J. Cell Sci. 114, 3047–3057
18. Bresnick, A. (1999) Curr. Opin. Cell Biol. 11, 26–33
19. Walsh, J., Gill, G. N., Rosenfeld, M. G., and Wells, A. (1991) J. Cell Biol. 114,
Epidermal Growth Factor Induces Fibroblast Contractility and Motility via a Protein Kinase C δ-dependent Pathway

Akihiro Iwabu, Kirsty Smith, Fred D. Allen, Douglas A. Lauffenburger and Alan Wells

J. Biol. Chem. 2004, 279:14551-14560.
doi: 10.1074/jbc.M311981200 originally published online January 27, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M311981200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 72 references, 36 of which can be accessed free at http://www.jbc.org/content/279/15/14551.full.html#ref-list-1