Protein Kinase C ζ Is Required for Epidermal Growth Factor–Induced Chemotaxis of Human Breast Cancer Cells

Ronghua Sun,[1] Ping Gao,[3] Lin Chen,[1] Dalong Ma,[2] Jiming Wang,[3] Joost J. Oppenheim,[3] and Ning Zhang[1,3]

[1]Department of Chemical Biology and State Key Laboratory of Molecular Dynamic and Stable Structures, College of Chemistry and [2]Laboratory of Medical Immunology, School of Basic Medical Science, Peking University, Beijing, China; and [3]Laboratory of Molecular Immunoregulation, Center for Cancer Research, Intramural Research Support Program, National Cancer Institute, Frederick, Maryland

Abstract

Chemotaxis plays an important role in cancer cell metastasis. In this study, we showed that epidermal growth factor (EGF) was a more potent chemoattractant than chemokine SDF-1α/CXCL12 for human breast cancer cell MDA-MB-231. Different inhibitors were used to evaluate the involvement of 12 protein kinase C (PKC) isotypes in the chemotactic signaling pathway. Chelerythrine chloride, an inhibitor of all PKC isotypes, blocked chemotaxis, whereas inhibitors of classic and novel PKC, such as Gö6976, Gö6850, or calphostin C, only impaired EGF-induced chemotaxis to a minor extent by ≯32% inhibition. These data suggested that atypical PKC were involved. The ligand-induced actin polymerization and cell adhesion were also similarly dependent on atypical PKC. Immunofluorescent staining showed an EGF-induced, LY294002-sensitive translocation of PKCζ from the cytosol to the plasma membrane, indicating that EGF was capable of activating PKCζ, probably via phosphoinositide 3 kinases. A myristoylated PKCζ pseudosubstrate blocked the chemotaxis with an IC_{50} of 20 μmol/L. To expand our investigation, we further showed that in MCF-7 and T47D, two additional human breast cancer cell lines, EGF-activated PKCζ and the PKCζ pseudosubstrate, inhibited chemotaxis. Taken together, our data suggest that PKCζ is an essential component of the EGF-stimulated chemotactic signaling pathway in human breast cancer cells. (Cancer Res 2005; 65(4): 1433-41)

Introduction

Chemotaxis, the directional movement of a cell in response to a chemical gradient, plays an important role in development, wound healing, angiogenesis, and immune responses (1–5). Recent studies indicate that chemotaxis contributes to the spread of cancer cells (6, 7). Blocking of a chemokine receptor, CXCR4, impaired the breast cancer metastasis to regional lymph nodes and the lungs (6). Numerous studies have revealed that the molecular mechanism of chemokine receptor–orchestrated chemotaxis can involve G-protein-coupled receptors (8–11). Binding of a chemokine activates G_i protein, resulting in a chain of downstream signaling events that include dissociation of heterotrimeric G proteins; activation of phosphoinositide 3 (PI3) kinase γ, Cdc42/Rac, and Arp2/3; and polymerization of actin at the leading edge of a cell (12–15).

Chemotaxis of breast cancer cells is also mediated by G-protein-independent receptors, such as epidermal growth factor (EGF) receptors, members of the receptor tyrosine kinase (RTK) family (16). In the presence of EGF, dimerization and autophosphorylation of EGF receptors induce the exposure of cytosolic binding sites to several parallel downstream signaling molecules, such as phospholipase C γ (PLCγ), Grb2/Sos, and PI3 kinases (17, 18). However, the precise molecular mechanism of EGF-elicited chemotaxis is largely unknown. Recent studies suggest that either PI3 kinase α or δ is required (19, 20). Blocking PLCγ with U73122 is reported to interfere with EGF-mediated breast cancer migration and tumor invasion (21–23). Cdc42 is required for directional movement of NR6 fibroblast and A431 cells (24). Actin polymerization elicited by EGF also plays a critical role in cell migration (25). These data indicate that although EGF may share some of the molecular components, such as Cdc42 and filamentous actin (F-actin), with chemokine-induced chemotaxis, it also utilizes a unique signal transduction pathway to direct cell migration.

Both G-protein-coupled receptors and RTK activate members of the protein kinase C (PKC) family, consisting of 12 serine/threonine kinases. The PKC family can be divided into three subfamilies based on their activation mechanism: classic PKC, such as PKC α, βI, βII, γ, which require both diacylglycerol and calcium for activation; novel PKC, such as δ, ε, θ, η, the activation of which requires diacylglycerol and not calcium; and atypical PKC, including ζ and λ, which do not need diacylglycerol or calcium for their activation (26, 27). PKC plays pleiotropic roles in cell polarity, migration, and adhesion. For instance, phosphorylation of integrin α6β4 by PKCα is associated with EGF-induced cell adhesion and migration (28). PKCα has also been shown to directly bind to β1 integrin (29). However, a recent study shows that phorbol 12-myristate 13-acetate–induced PKCα activation results in a decrease in EGF-induced cell motility in MDA-MB-231 cells (30). PKCε has also been shown to be colocalized with integrin β1 and to contribute to cell motility (31). PKCδ is required for EGF-induced phosphorylation of myosin light chain, a key step in forming contractile ring during migration (32). PKCζ has been indicated to play an important role in determining cell polarity (33–37). Studies using isozyme-specific inhibitors suggest that PKCζ is also involved in chemokine-triggered cell adhesion and actin assembly in polymorphonuclear cells, presumably mediated by G_i protein (38, 39). However, the role of PKCζ in RTK-mediated chemotaxis has not been characterized. In the current study, we investigated the roles of PKC isotypes in EGF-induced chemotaxis and found that PKCζ was the only essential PKC isotype.

Materials and Methods

Cell Culture and Reagents. Human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Manassas, VA). T47D and MCF7 were from Invitrogen (Carlsbad, CA). They were cultured

Requests for reprints: Ning Zhang, Department of Chemical Biology and State Key Laboratory of Molecular Dynamic and Stable Structures, College of Chemistry, Peking University, Beijing 100871, P.R. China. Phone: 86-10-62755154; Fax: 86-10-62751708; E-mail: zhangnz@pku.edu.cn.

in RPMI 1640 with 10% FCS (complete medium). Chemotaxis chambers and membranes were purchased from Neuroprobe (Gaithersburg, MD). Human EGF was from BD Biosciences (San Jose, CA). Fibronectin (0.1%) was from Sigma (St. Louis, MO). LY294002, U73122, AG1478, and AG17 were from Calbiochem (La Jolla, CA). Calphostin C, Gö6850, Gö6976, and chelerythrine chloride were from Alexis Biochemicals (San Diego, CA). Polyclonal antibodies against PKCζ (sc-216), PKCα/βI/βII (c-20), and a monoclonal anti-human EGF receptor (EGFR) inhibitory antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-human EGFR polyclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Oregon Green 514 phalloidin was from Molecular Probes, Inc. (Eugene, OR). Myristoylated and nonmyristoylated pseudopeptides for PKCζ were synthesized by Calbiochem based on the reported sequence from the pseudosubstrate region of human PKCζ (SIYRRGARRWRKL). Myristoylated scrambled peptide (RLYRKRIWRSAGR) was synthesized by GL Biochem (Shanghai) Ltd. (Shanghai, China). Peptides were solubilized immediately before use at a 1 mM concentration in PBS, pH 7.2.

Chemotaxis Assay. Chemotaxis assays were done as described by the manufacturer (Neuroprobe, Cabin John, MD) and by Zhang et al. (40). Briefly, chemoattractants were loaded into the lower chemotaxis chamber. MDA-MB-231 cells (0.5×10^6 cells/mL), suspended in binding medium (RPMI 1640, 0.1% bovine serum albumin, and 25 mM HEPES), were then added into the upper chambers. The two chambers were separated by a 10 μm filter that had been pretreated with 0.001% fibronectin in RPMI 1640 at 4°C overnight. The chambers were incubated in 5% CO_2 at 37°C for 3 hours. The filter was then washed, fixed, and stained. The number of migrated cells was counted in three high-power fields (400×). The chemotaxis index was calculated as the ratio of the number of cells responding to a chemoattractant gradient over the number of migrated cells in a medium control. For checkerboard assay, MDA-MB-231 cells were mixed with different concentrations of EGF before adding to upper wells of the chamber. For inhibition by anti-EGFR inhibitory antibody, MDA-MB-231 cells were preincubated with indicated concentrations of anti-EGFR antibody or isotype-matched mouse IgG2a at room temperature for 15 minutes. For the inhibitor assay, MDA-MB-231 cells were pretreated with inhibitors at the indicated concentration for 45 to 60 minutes at 37°C and then loaded into the upper chamber. Statistical analysis was carried out to determine the significance of chemotactic response using PRIZM 3, ANOVA analysis.

Protein Kinase C Translocation Assay. Translocation of PKC in MDA-MB-231 cells was analyzed using immunofluorescent staining techniques with confocal microscopic analysis (40). In brief, cells were cultured 48 hours before the experiment then starved in binding medium for 3 hours followed by stimulation with 10 ng/mL EGF at 37°C for 5 to 10 minutes before fixation with 4% formaldehyde. For inhibitor control, cells were treated with 50 μmol/L of LY294002 at 37°C for 1 hour before EGF stimulation. Cells were then permeabilized with 0.2% Triton X-100 in buffer (10 mM HEPES, 20 mM KH_2PO_4, 5 mM EGTA, 2 mM $MgCl_2$, Dulbecco's PBS, pH 6.8) and stained with polyclonal antibodies to various PKC isotypes followed by probing with FITC-labeled goat anti-rabbit antibody. Cells were visualized using a Zeiss LSM 410 inverted fluorescent confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Western Blotting Assay. Western blotting assays were carried out as described by Zhang et al. (40). Proteins (20 μg per lane) were separated by 8% SDS-PAGE, transferred onto polyvinylidene difluoride membranes (Pierce, Rockford, IL), probed with rabbit anti-EGFR or anti-PKCζ (1:1000) antibodies, and visualized using enhanced chemiluminescence reagents. In Akt/PKB and extracellular signal-regulated kinase (ERK) 1/2 activation assay, MDA-MB-231 cells were activated by 10 ng/mL EGF for 5 minutes in the presence or absence of inhibitors. Then, the cells were lysed for Western blotting analysis with the use of antiphosporylated Akt/PKB, Akt/PKB, phosphorylated ERK1/2, and ERK1/2 antibodies.

Flow Cytometry Assay. Cell surface expression level of EGFR was monitored by using a flow cytometer [fluorescence-activated cell sorter (FACS)]. MDA-MB-231, MCF-7, or T47D cells (5×10^5 cells per sample) were washed twice with ice-cold FACS buffer (Dulbecco's PBS, 1% FCS, 5 mM EDTA, and 0.1% sodium azide, pH 7.4). The cells were stained with anti-EGFR monoclonal antibody for 30 minutes on ice. After three washes with ice-cold FACS buffer, FITC-labeled secondary antibody was applied for another 30 minutes on ice. The cells were washed twice and analyzed immediately by flow cytometry (BD Biosciences). For experiment with synthesized PKCζ pseudosubstrate peptides, MDA-MB-231 cells were pretreated with 50 μmol/L myr-pseudosubstrate, 50 μmol/L non–myr pseudosubstrate, or medium alone at 37°C for 45 minutes. The cells were washed twice with ice-cold FACS buffer before immunostaining.

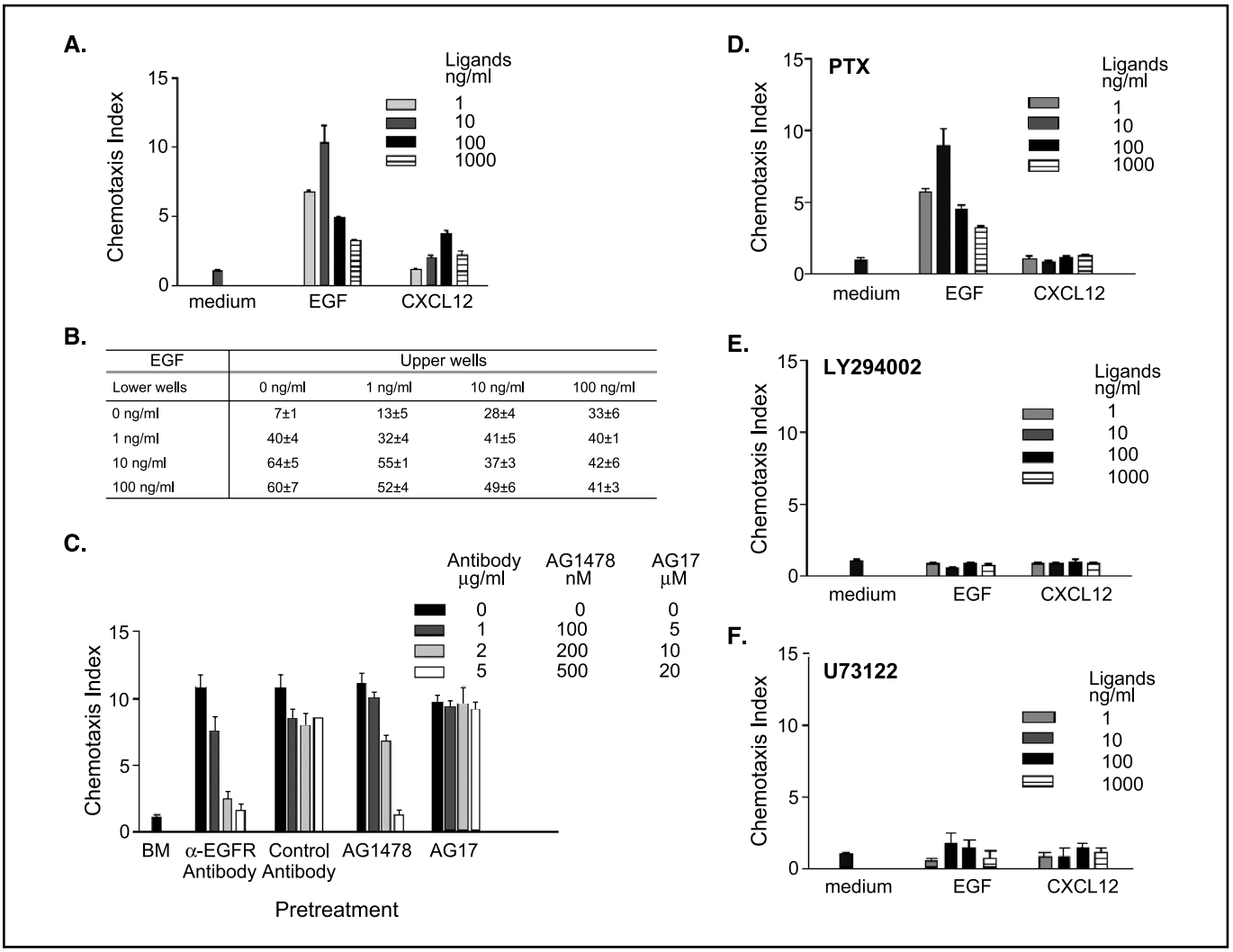
F-Actin Content Assay. F-actin was quantified by methanol extraction of Oregon Green 514/phalloidin–stained cells as described previously (25). Briefly, MDA-MB-231 cells were plated and cultured for 18 hours in complete medium followed by further culturing in binding medium for 2 hours. Cells were then treated with the indicated inhibitors at specific concentrations or binding medium for another hour followed by 50 ng/mL EGF stimulation at 37°C for the indicated period of time. Cells were fixed, permeabilized, and stained in the dark with Oregon Green 514 phalloidin diluted in F-buffer (10 mM HEPES, 20 mM KH_2PO_4, 5 mM EGTA, 2 mM $MgCl_2$, Dulbecco's PBS, pH 6.8) at room temperature for 30 minutes. After five washes, bound phalloidin was extracted with methanol at 4°C for 90 minutes and subjected to fluorescence analysis at 511 nm excitation and 529 nm emission. At the same time, an aliquot of cells were analyzed by a bicinchoninic acid assay (Pierce) to determine total protein in the sample. Fluorescence signals were normalized against total protein. Results were expressed as relative F-actin content, where

$$F-actin\,\Delta t/F-actin0 = (fluorescence\,\Delta\,t/mg\ per\ mL)/(fluorescence\ 0/mg\ per\ mL)$$

Adhesion Assay. Glass coverslips were coated with 10 μg/mL fibronectin in serum-free RPMI 1640 for 2 hours at 37°C and air-dried before use. Monolayer of MDA-MB-231 cells were trypsinized for 5 minutes at room temperature and stopped with culture medium, pelleted, and resuspended to 2.7×10^5 cells/mL in complete medium. The cell suspension was placed in a 37°C 5% CO_2 incubator for 20 minutes before the adhesion assay. Cells were treated at 37°C for 45 minutes with inhibitors at the indicated concentrations or with medium alone. EGF or medium (10 ng/mL) was added to each of the cell suspensions containing inhibitors or medium control. EGF-treated or nontreated suspension (1.5 mL) was then immediately added to the appropriate 35 mm dish containing the slide. Cells were allowed to attach for 5 minutes before gently washing and fixing the slides. The number of attached cells was counted by light microscopy (200×).

Results

Using gradients of EGF or SDF-1α/CXCL12, we compared the chemotactic effect of EGFR with CXCR4, a chemokine receptor. Consistent with previous reports, SDF-1α/CXCL12, a specific ligand for CXCR4, induced the chemotaxis of MDA-MB-231 cells in a dose-dependent manner (Fig. 1*A*; ref. 6). EGF-induced chemotaxis also exhibited a typical bell-shaped dose-response curve and the chemotaxis indexes were as much as 3-fold more than those induced by CXCL12. Checkerboard analysis indicated that EGF also induced chemokinesis, an indication of enhanced cell motility (Fig. 1*B*). As shown in Fig. 1*C*, the inhibitory antibody to EGFR blocked the chemotaxis in a dose-dependent manner, indicating that the chemotactic activity of EGF requires EGFR (23). Furthermore, AG1478 (0.5 μmol/L), a specific inhibitor of EGFR activation, also inhibited chemotaxis, suggesting that EGFR dimerization and autophosphorylation is required (23).



B.

EGF	Upper wells			
Lower wells	0 ng/ml	1 ng/ml	10 ng/ml	100 ng/ml
0 ng/ml	7±1	13±5	28±4	33±6
1 ng/ml	40±4	32±4	41±5	40±1
10 ng/ml	64±5	55±1	37±3	42±6
100 ng/ml	60±7	52±4	49±6	41±3

Figure 1. EGF-induced chemotaxis of MDA-MB-231 cells. *A*, comparison of chemotactic dose-response of MDA-MB-231 cells to EGF, CXCL12 ($P < 0.0027$). *B*, chemokinesis of MDA-MB-231 cells induced by EGF. MDA-MB-231 cells were mixed with indicated concentrations of EGF or medium only before immediate application to upper chamber. *C*, dose-dependent inhibition of EGF-induced MDA-MB-231 cell migration was achieved by anti-EGFR antibody ($P < 0.0041$) and EGFR-specific inhibitor AG1478 ($P < 0.0072$). MDA-MB-231 cells (0.5×10^6 cells/mL) were incubated with 1, 2, and 5 μg/mL of anti-EGFR-specific antibody or isotype-matched mouse IgG2a at room temperature for 15 minutes and their chemotactic response to EGF at 10 ng/mL was determined. *D*, effect on chemotactic dose-response to EGF or CXCL12 stimulation following treatment with pertussis toxin at 150 ng/mL ($P < 0.0051$, two-way ANOVA). *E*, effect on chemotactic dose-response to EGF or CXCL12 stimulation following treatment with PI3 kinase inhibitor LY294002 at 30 μmol/L. *F*, effect on chemotactic dose-response upon EGF or CXCL12 stimulation following treatment with 2 μmol/L U73122. Chemotaxis assay was done as described in Materials and Methods. *Columns,* mean of triplicate measurements; *bars*, SD. Chemotaxis data were analyzed by one-way or two-way ANOVA.

To assess whether these two types of receptors share certain chemotactic signaling components, we examined the effects of pertussis toxin, LY294002, and U73122. Pretreatment with 150 ng/mL pertussis toxin, a specific inhibitor of G_i protein, irreversibly inhibited SDF-1α/CXCL12-induced chemotaxis (Fig. 1*D*) but not EGF-induced chemotaxis. In the presence of 30 μmol/L LY294002, a specific PI3 kinase inhibitor, both SDF-1α/CXCL12 and EGF-induced chemotaxis were inhibited, suggesting that each required PI3 kinases (Fig. 1*E*; refs. 19, 20). U73122 (2 μmol/L), a specific inhibitor of PLC, also blocked the chemotactic effect induced by either SDF-1a/CXCL12 or EGF (Fig. 1*F*; refs. 21–23). Overall, our data showed that EGF was a more potent chemoattractant for MDA-MB-231 cells than CXCL12 and suggested that EGFR-mediated chemotaxis required PI3 kinases and was G_i protein independent.

To assess the role of PKC family members in EGFR-mediated chemotaxis, we investigated the inhibitory effects of a spectrum of specific PKC inhibitors. Chelerythrine chloride, a specific inhibitor that inhibits all the PKC isotypes with an IC_{50} of 660 nmol/L, blocked EGF-induced chemotaxis in a dose-dependent manner (Fig. 2*A*). The combination of chelerythrine and LY294002 also inhibited chemotaxis (data not shown). However, in the presence of inhibitors of classic or novel PKC, EGF still elicited marked chemotaxis. Gö6976, a specific inhibitor for classic PKC isotypes with an IC_{50} value ranging from 2.3 to 6 nmol/L, exhibited <20% inhibition at a concentration of 50 nmol/L (Fig. 2*A*). Calphostine C, a specific inhibitor for diacylglycerol binding site, inhibited by 32% without any dose-dependence (Fig. 2*A*). Gö6850, another specific inhibitor for classic and novel PKC, inhibited chemotaxis only by 18% at 100 nmol/L (Fig. 2*A*). Thus, classic or novel PKC isotypes

arenot essential for EGF-elicited chemotaxis. To confirm these observations, we assessed the effect of all these PKC inhibitors at doses of 1 to 100 ng/mL EGF. Once again, only treatment with chelerythrine chloride completely blocked EGF-elicited chemotaxis (Fig. 2*B*). Therefore, this data suggests that an atypical PKC might be required for EGF-elicited chemotaxis.

Actin polymerization, a chemoattractant-induced response, provides an established indicator for the presence of a chemotactic signal. To confirm the results of the chemotaxis assays, a ligand-stimulated actin polymerization assay in the presence of selective inhibitors was done. Both EGF and SDF-1α/CXCL12 induced a transient polymerization of globular actin, consistent with a previous report (Fig. 3*A*; ref. 25). Furthermore, we detected similar EGF-induced actin polymerization in MCF-7 and T47D, two additional human breast cancer cell lines (data not shown). Our data also suggests that the EGF- and SDF-1α/CXCL12–induced signal transduction pathways converged at the point of actin polymerization. In the presence of 30 μmol/L LY294002 or 2 μmol/L U73122, actin polymerization elicited by 10 ng/mL EGF was significantly reduced (Fig. 3*B*), consistent with our chemotaxis data in Fig. 1*D* and *E*. Treatment with chelerythrine chloride also inhibited actin polymerization, confirming that PKC was required for EGF-induced chemotaxis (Fig. 3*B*). Chelerythrine chloride treatment also caused a decrease in the basal level of F-actin (data not shown). In the presence of Gö6850, EGF still induced actin polymerization, further suggesting that classic and novel PKC isotypes were not essential for chemotaxis (Fig. 3*B*). Taken together, our data implicates an atypical PKC in EGF-induced actin polymerization. Furthermore, the EGF-induced chemotaxis signaling pathway may converge with the G-protein-mediated pathway at or upstream of actin polymerization.

Adhesion is closely associated with cell migration and is another function frequently regulated by PKC. As shown in Fig. 3*C*, 10 ng/mL EGF induced a rapid increase in cell adhesion to fibronectin surfaces. Gö6850 and Gö6976 did not interfere with the basal or EGF-stimulated cell adhesion. LY294002, U73122, and chelerythrine chloride each blocked EGF-stimulated rapid adhesion, indicating that PI3 kinases, PLC, and an atypical PKC were involved. Treatment with chelerythrine C also inhibited basal adhesion, suggesting that an atypical PKC may also play a role in EGF-independent cell adhesion.

Because both actin and chemotaxis assays suggested that an atypical PKC might be involved in EGF-induced chemotaxis, we investigated whether EGF was capable of activating PKCζ, an atypical PKC that has been implicated in modulating cytoskeleton structure. Subcellular redistribution is a hallmark of PKC activation (26). Using immunohistochemical staining, we examined the distribution of PKCζ on EGF stimulation. As shown in Fig. 4*A*, PKCζ staining was distributed in the cytosol region of a resting cell. In the presence of 10 ng/mL EGF, the fluorescent signals were redistributed to the plasma membrane region of ~34% of the MDA-MB-231 cells, suggesting that EGF

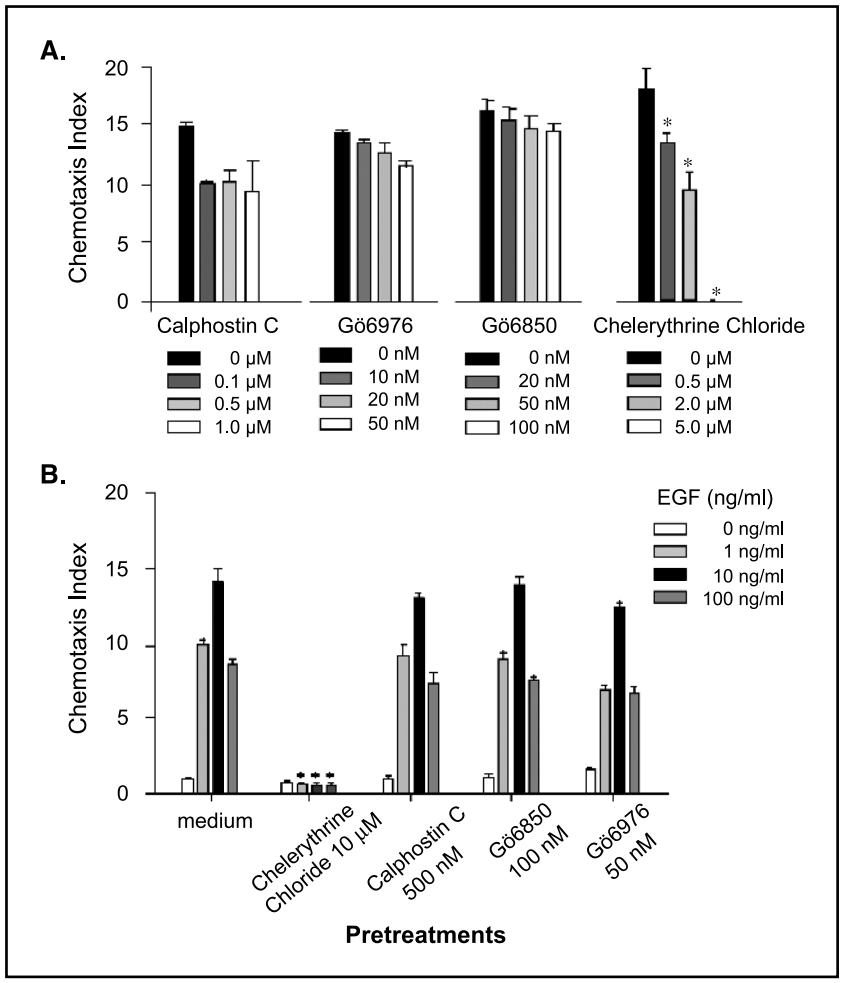


Figure 2. Effects of PKC inhibitors on EGF-induced chemotaxis. *A*, effect on chemotaxis of calphostin C, an inhibitor of diacylglycerol binding, 0-1.0 μmol/L; Gö6976, an inhibitor of classic PKC, 0-50 nM; Gö6850, an inhibitor of both classic and novel PKC, 0-100 nM; and chelerythrine chloride, an inhibitor of all PKC, 0-5.0 μmol/L ($P < 0.0012$). Cells were stimulated with 10 ng/mL EGF following inhibitor treatment. *B*, effect on chemotactic dose-response of different inhibitors. Cells were pretreated with chelerythrine chloride (10 μmol/L), calphostin C (500 nmol/L), Gö6850 (100 nmol/L), and Gö6976 (50 nmol/L) before stimulation with EGF at various concentrations (0-100 ng/mL; $P < 0.0002$). Chemotaxis assay was done as described in Materials and Methods. *Columns,* mean of triplicate measurements; *bars,* SD. Chemotaxis data were analyzed by two-way ANOVA.

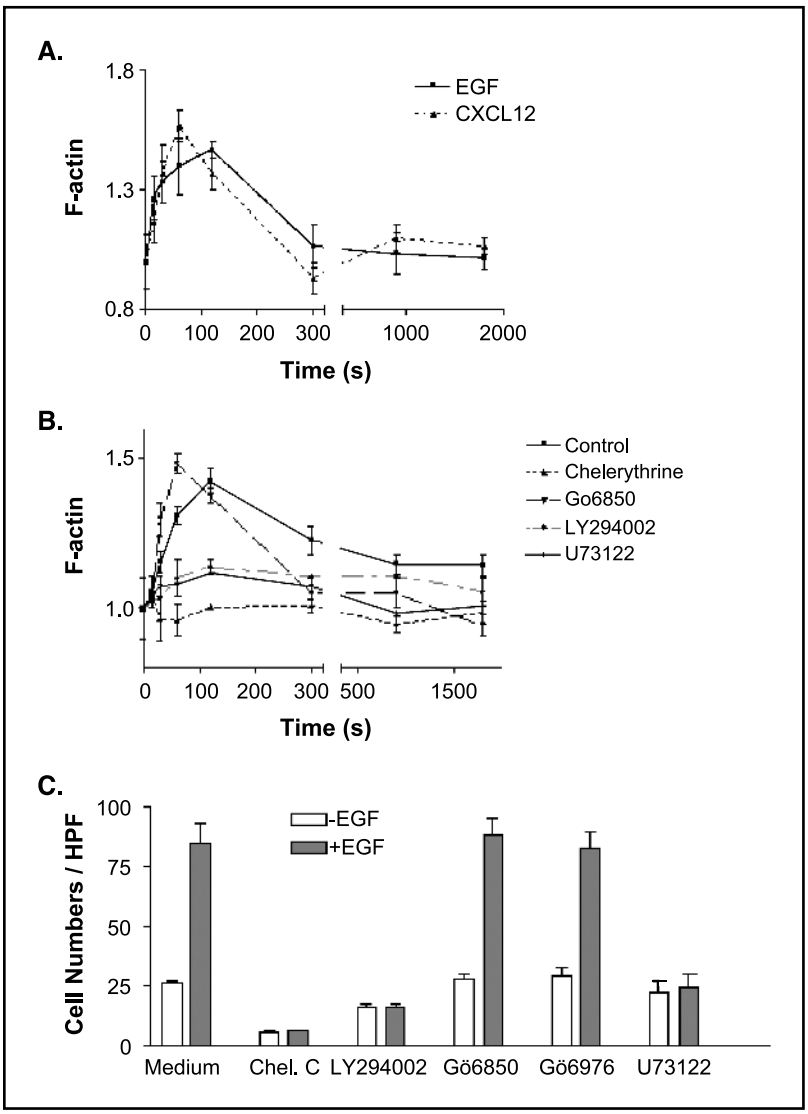


Figure 3. Actin polymerization and cell adhesion of EGF-stimulated MDA-MB-231 cells. *A*, time course of relative F-actin content in MDA-MB-231 cells following EGF and CXCL12 stimulation. EGF, 50 ng/mL; CXCL12, 100 ng/mL. *B*, time course of relative F-actin content in MDA-MB-231 cells following EGF stimulation with and without different inhibitors. EGF, 50 ng/mL; chelerythrine chloride, 10 μmol/L; Gö6850, 100 nM; LY294002, 30 μmol/L; U73122, 2 μmol/L. F-actin content was determined as described in Materials and Methods. *Points,* mean of triplicate measurements; *bars,* SD. *C*, effect of EGF stimulation with and without different inhibitors on adhesion of MDA-MB-231 cells. EGF, 10 ng/mL; chelerythrine chloride, 10 μmol/L; LY29002, 30 μmol/L; Gö6850, 100 nM; Gö6976, 50 nM; U73122, 2 μmol/L. Adhesion assay was done as described in Materials and Methods. *Columns,* mean of triplicate measurements; *bars,* SD. The adhesion data were analyzed by one-way ANOVA ($P < 0.007$).

activated PKCζ. In the presence of LY294002, this EGF-induced translocation of PKCζ was impaired, suggesting that PKCζ exerts its function downstream of PI3 kinases in the chemotaxis signaling pathway. PKCα has been suggested to play a role in EGF-mediated chemotaxis. However, we were not able to detect a clear redistribution of cytosolic PKCα to the cytoplasmic membrane upon EGF stimulation. We further examined whether EGF activated PKCζ of other human breast cancer cell lines. In 5 minutes, EGF at 10 ng/mL stimulated a similar redistribution of cytosolic PKCζ to cell membrane in MCF-7 and T47D as in MDA-MB-231 cells (Fig. 4*B*). The translocation, sensitive to LY294002 interference, occurred in 25% of MCF-7 and 32% of T47D cells (data not shown). It seemed that cytosolic PKCζ close to the cell membranes was among the first to be depleted. Thus, immunohistochemical data showed that EGF was capable of activating PKCζ, an atypical PKC in MDA-MB-231 cells, and the activation process was PI3 kinase dependent.

Akt/PKB, a pivotal effector directly downstream of PI3 kinases, had been implicated in G-protein-coupled receptor–mediated chemotaxis (41). EGF induced Akt/PKB activation, as shown by an increase in phosphorylated Akt/PKB (Fig. 4*C*). Upon adding Akt inhibitor III, phosphorylation of Akt decreased up to 70%, indicating the inhibitor was functional (42). In the presence of Akt inhibitor III, EGF-induced chemotaxis was significantly impaired in a dose-dependent manner, suggesting that Akt was involved in EGF-induced chemotaxis (Fig. 4*C*). We further examined the role of ERK1/2 in chemotaxis. PD98059, a specific inhibitor of ERK, induced a marked decrease in ERK1/2 phosphorylation but had no detectable effect on EGFR-mediated chemotaxis. Thus, our data suggested that EGF-induced Grb2-Sos-ERK1/2 pathway did not play a role in chemotaxis, consistent with previous report (23).

Finally, we used a PKCζ pseudosubstrate, a proven specific inhibitor, to confirm the involvement of this molecule in EGF-induced chemotaxis (38, 39). Myristoylation of this pseudosubstrate was required for delivering this peptide through the cytoplasmic membrane. In the presence of myristoylated peptide, EGF-induced chemotaxis was inhibited in a dose-dependent manner, with an apparent IC_{50} of 20 μmol/L (Fig. 5*A*). There was no detectable change in cell motility or proliferation rate after

addition of the peptide (data not shown). In controls, the nonmyristoylated peptide at 50 μmol/L caused no significant inhibition. Furthermore, the scrambled pseudopeptide did not impair chemotaxis, confirming the specificity of the pseudosubstrate inhibitor in use. To extend our investigation, two more human breast cancer cell lines, MCF-7 and T47D, were examined for their EGF-induced chemotaxis. As shown in Fig. 5*B*, EGF elicited a typical bell-shaped chemotactic response of these two cell lines, which was totally inhibited by myristoylated pseudosubstrate at 50 μmol/L. We next examined the expression pattern of PKCζ. Western blotting data clearly showed robust expression of PKCζ protein in all three human breast cancer cell lines, consistent with their critical role in chemotaxis (Fig. 5*C*). One potential pitfall of the inhibitor experiments is that PKCζ inhibitors down-regulates cell surface EGFR, resulting in a decrease in chemotaxis index. Western blotting and FACS analysis showed a considerable expression of EGFR on the surface of all three human breast cancer cells (Fig. 5*D* and *E*). Treatment with myristoylated pseudosubstrate did not induce any detectable loss of surface EGFR, suggesting that PKCζ pseudosubstrate is not involved in regulating the expression level of surface EGFR (Fig. 5*E*). Taken together, our results indicated that PKCζ is required for EGF-induced chemotaxis of human breast cancer cells.

Discussion

Accumulating reports have shown that PKCζ plays pleiotropic roles in multiple signal transduction pathways, such as mitogen-activated kinase cascade, NF-κB activation, ribosomal S6-protein kinase signaling, and cell polarity pathway (26, 33). Our data suggest that PKCζ exhibits the novel function of mediating RTK-elicited chemotaxis. Previous reports have suggested that PKCα and PKCε are involved in EGF-induced chemotaxis (28–31). They enhance cell migration by directly phosphorylating integrins. It was even suggested that a phorbol 12-myristate 13-acetate gradient maybe sufficient to account for EGF-mediated cell migration (29). Our data

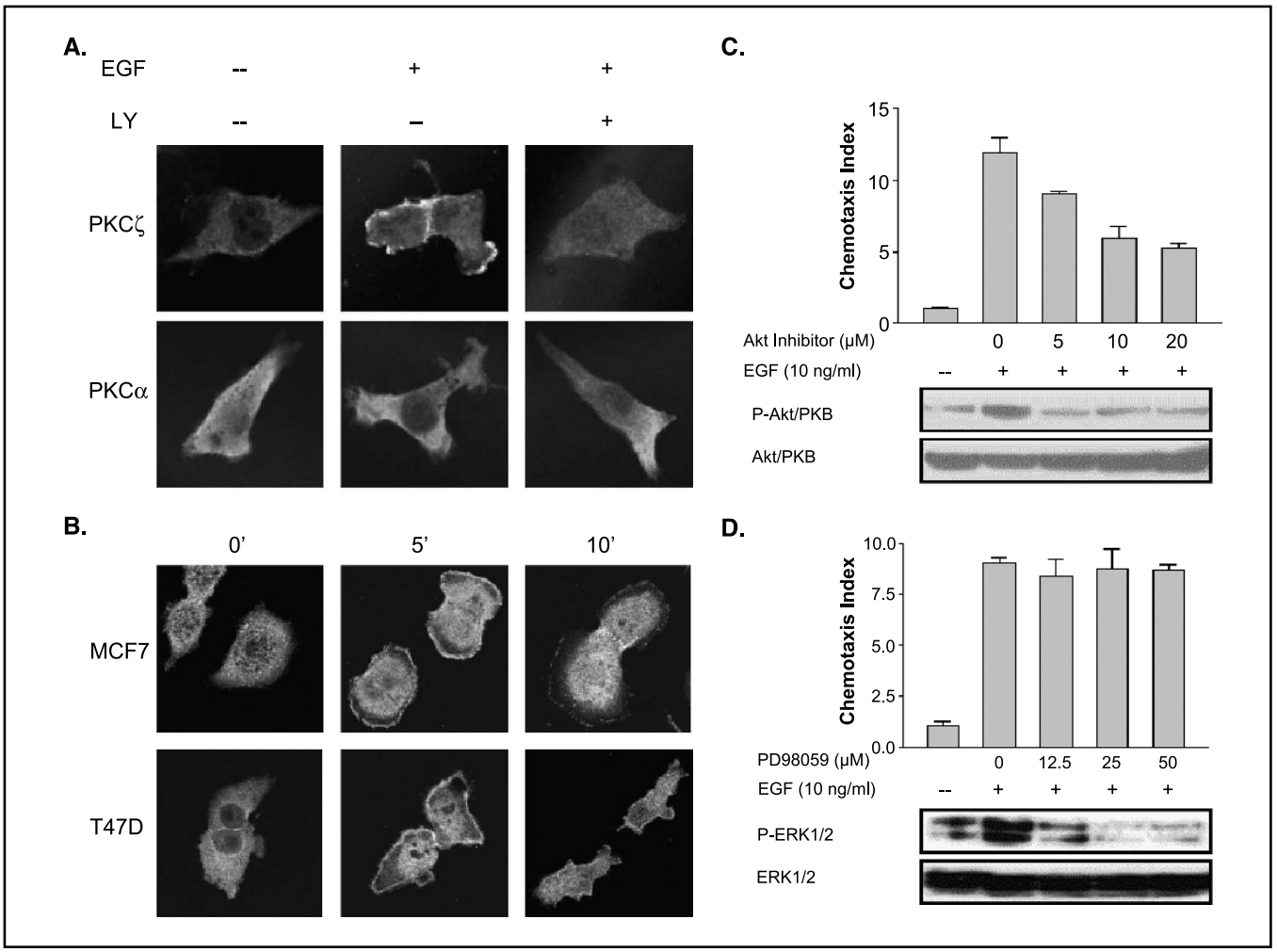


Figure 4. Involvement of PKCζ in EGF-induced cellular responses of MDA-MB-231 cells. *A*, cellular distribution of PKCζ and PKCα before and after EGF stimulation for 10 minutes. EGF, 10 ng/mL; LY294002, 50 μmol/L. Translocation assay was done as described in Materials and Methods. *B*, cellular distribution of PKCζ in MCF-7 and T47D cells at time 0, 5, and 10 minutes after 10 ng/mL EGF stimulation. *C*, in the presence of Akt inhibitor III at 0, 5, 10, and 20 μmol/L, the phosphorylation of Akt/PKB– and EGF-induced chemotaxis were decreased. The total input of Akt was consistent ($P < 0.002$). *D*, in the presence of PD98059 at 0, 12.5, 25, and 50 μmol/L, the phosphorylation of ERK1/2 is decreased. There was no detectable change in chemotaxis response induced by 10 ng/mL EGF. Total input of ERK1/2 was the same. (The chemotaxis data were analyzed by one-way ANOVA.)

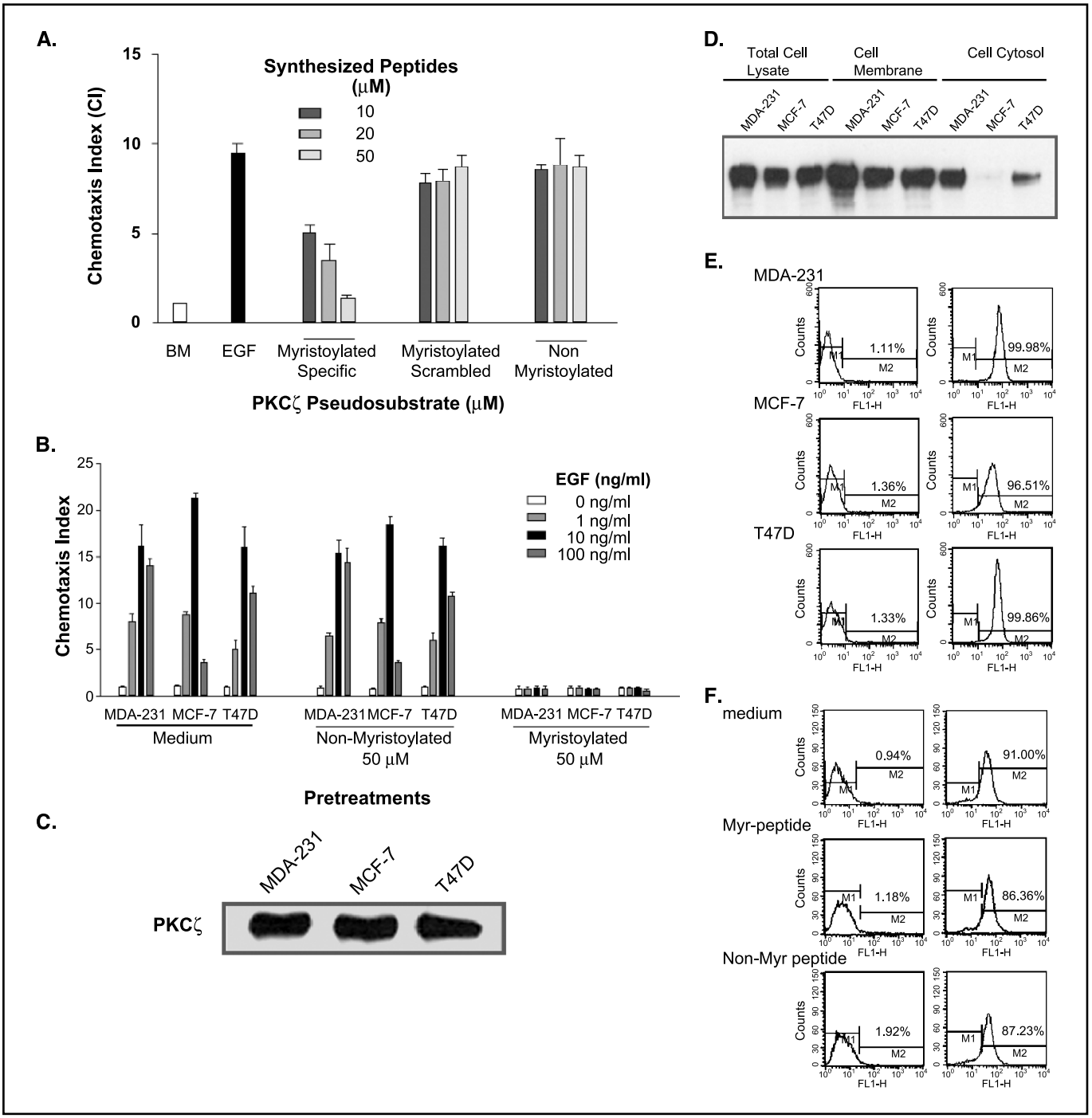


Figure 5. *A*, blockage of MDA-MB-231 cells chemotaxis in response to EGF by myristoylated PKCζ pseudosubstrate peptide (myr-peptide). MDA-MB-231 cells (0.5×10^6 cells/mL) were incubated with 10, 20, and 50 μmol/L of myristoylated PKCζ pseudosubstrate peptide, myristoylated PKCζ scrambled peptide, nonmyristoylated PKCζ pseudosubstrate peptide, or PBS (solvent for synthesized peptide) at 37°C for 45 minutes. Then, the cells were measured for their chemotactic response to 10 ng/mL EGF or medium only. The results were shown as chemotactic index (±SD). *Columns,* mean of triplicate measurements; *bars,* SD. *B*, inhibitory effects of 50 μmol/L PKCζ pseudosubstrate on MCF-7 and T47D human breast cancer cells. *Columns,* mean of triplicate measurements; *bars,* SD. Chemotaxis data were analyzed by one-way ANOVA ($P < 0.021$). *C*, expression level of PKCζ subtypes in MDA-MB-231, MCF-7, and T47D cells. Three breast cancer cell lines were collected separately and lysed. Twenty micrograms of cell lysates for each sample were subjected to 8% SDS-PAGE and probed with specific rabbit anti-PKCζ polyclonal antibody. *D*, Western blotting analysis of EGFR expression on MDA-MB-231, T47D, and MCF-7 cells. Fifty micrograms of protein was loaded on each lane of SDS-PAGE. *E*, cell surface expression level of EGFR on MDA-MB-231, MCF-7, and T47D cells. Cells (0.5×10^6) of each cell line were collected and washed with FACS buffer. The cells were then stained with anti-EGFR monoclonal antibody or isotype-matched mouse IgG2a followed by staining with FITC-conjugated rabbit anti-mouse antibody. *F*, effect of pretreatment with of PKCζ pseudosubstrates on surface expression of EGFR by MDA-MB-231 cells. MDA-MB-231 cells (0.5×10^6 cells/mL) were incubated with 50 μmol/L of myristoylated-PKCζ pseudopeptide, nonmyristoylated pseudopeptide, or PBS only at 37°C for 45 minutes. Then, the cells were washed twice with cold FACS buffer followed by immunostaining with anti-EGFR-specific monoclonal antibody or isotype-matched mouse IgG2a. Data showed percentage of positive staining cells.

argue that among various subtypes of PKC activated by EGF, PKCζ plays an essential role in chemotaxis. The inhibitors of classic and novel PKC, such as Gö6976, Gö6850, or calphostine C, only impaired EGF-elicited chemotaxis to a minor extent. This minor inhibitory effect is probably caused by the blockade of PKCα or ε, resulting in an impairment in EGF-induced phosphorylation of integrin (29, 31). To examine whether a gradient of PKC activation accounts for the driving force of chemotaxis, we measured the chemotaxis index of MDA-MB-231 cells in the presence of a phorbol 12-myristate 13-acetate gradient. We were not able to detect a significant chemotaxis in response to phorbol 12-myristate 13-acetate at 0.02 to 2000 nmol/L (data not shown). When all 12 PKC subtypes were blocked by chelerythrine chloride, a specific inhibitor that interferes with the catalytic motif of all PKC, EGF-elicited chemotaxis was totally impaired, indirectly suggesting that atypical PKC isotypes may be the effectors participating in chemotaxis. Actin polymerization and cell adhesion assays further confirmed the essential role of atypical PKC in EGFR-mediated chemotaxis. The fact that EGF induced the translocation of PKCζ to the plasma membrane directly indicates that EGF is capable of activating PKCζ in MDA-MB-231 cells. Finally, the myristoylated pseudosubstrate of PKCζ, a proven specific inhibitor, blocked EGF-induced chemotaxis in a dose-dependent manner. Thus, of the 12 isozymes, PKCζ is uniquely required for EGF-mediated chemotaxis. We were able to confirm these observations in MCF-7 and T47D, two additional human breast cancer cell lines, indicative of a general role of PKCζ in human breast cell chemotaxis.

The molecular mechanism that governs EGF-induced PKCζ activation still needs to be further elucidated. Immunofluorescent staining data suggests that PI3 kinases are required for PKCζ activation. Furthermore, inhibiting Akt/PKB, a signaling component immediately downstream of PI3 kinases, impairs chemotaxis up to 50%. The lack of complete inhibition may indicate that activation of a small percentage of Akt/PKB is sufficient for chemotaxis or that an unidentified signaling molecule, downstream of PI3 kinases, shares partially redundant function with Akt/PKB. Because PKCζ does not contain a pleckstrin homology domain and cannot be directly activated by PI3 kinases, we speculate that its activation relies on a pleckstrin homology domain–containing protein, such as Akt/PKB. Further analysis of the relationship between Akt/PKB and PKCζ is under way by using siRNA that targets Akt/PKB. In the light of recent reports and our data, we propose three possible models explaining the mechanism of PKCζ activation by PI3 kinases (34–39). One pathway involves activation of PI3 kinases that further stimulates Cdc42. Consequently, PKCζ/Par6 complex is recruited to the plasma membrane (34, 35). Second, it has been shown that, upon activation by RhoA, PKCζ is translocated to the plasma membrane and helps to enhance the lateral motility of LFA-1 in lymphocytes (39). Thus, it is also possible that EGF induced PKCζ activation through Rho. The third possibility is that phosphorylated PKCζ binds to membrane-associated PDK1, a kinase that is recruited by the products of PI3 kinases (33). Studies on the translocation of PKCζ-GFP using a PKCζ-GFP/MDA-MB-231 cell line would further clarify pathway involved in EGF-induced chemotaxis. These studies are currently in progress.

Our data suggest that RTK- and G-protein-coupled receptor–mediated chemotaxis signaling pathways converge at or upstream of PKCζ. In chemokine-induced chemotaxis, $G_{\beta\gamma}$ dissociated from $G_{\alpha i}$ activates PI3 kinase γ and PLCβII. PLCβII indirectly activates classic and novel PKC. However, PLCβII and novel or classic PKC do not play a significant role in G_i-protein-mediated chemotaxis. Extensive research using wortmannin, LY294002, and *pi3kγ−/−* mice has shown that PI3 kinase γ is essential in the signaling pathway (14, 15). Its activity plays an important role in recruiting the downstream signaling molecules to the leading edge of a migrating cell (43). Recently, PI3 kinase γ has been shown to mediate lymphocyte adhesion and migration by regulating Rho and PKCζ (39). EGFR seems to use different signaling molecules for chemotaxis. Upon ligand binding, EGF receptors dimerize to activate a spectrum of downstream signaling molecules, including Src homology and collagen protein, Janus-activated kinase 1, PI3 kinases, and PLCγ (17, 18). The fact that pertussis toxin does not interfere with EGF-elicited chemotaxis clearly rules out the involvement of G_i-protein. However, similar to G_i-mediated chemotaxis, activation of PKCζ has been shown to be required for EGF-elicited human breast cancer cell chemotaxis, and the activation of PKCζ seems to require PI3 kinase as well. We speculate that PKCζ is activated by PI3 kinase α or subtypes. Taken together, our data suggest that although G-protein-coupled receptors and EGFR orchestrate different upstream signaling components, their pathways in mediating chemotaxis seem to converge at or before PKCζ. Furthermore, because many RTK share similar signal transduction pathways, we further speculate that PKCζ may be required for chemotaxis by other RTK.

Identification of PKCζ as a convergence point of EGFR and chemokine receptor–mediated chemotaxis provides us with a potential new target for anticancer drugs. Blocking CXCR4 with antibodies effectively down-regulates cancer cell metastasis (6). However, there are many other receptors capable of inducing chemotaxis, such as CXCR1/2 and EGF. In fact, our data show that EGF induces a greater chemotactic response than SDF-1α by MDA-MB-231 cells. Thus, targeting one particular receptor may be insufficient to prevent cancer cell migration. Because PKCζ is required by both EGFR and chemokine receptor–mediated chemotaxis, it might be an effective target for more potent antimetastasis therapeutic strategies. Because the PKCζ-deficient mice are grossly normal, pharmacologic inhibition of this enzyme should not be lethal for the hosts (44). We postulate that blocking PKCζ may completely impair the chemotactic activities of some cancer cells, resulting in a decrease in tumor invasion and spreading.

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