Distinct Mechanisms Mediate the Initial and Sustained Phases of Cell Migration in Epidermal Growth Factor Receptor-Overexpressing Cells

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Abstract

Elevated levels of epidermal growth factor receptor (EGFR) are predictive of increased invasion and metastasis in many human cancers. In the present study, we have shown that two distinct pathways regulate cell migration in EGFR-overexpressing invasive cells such as MDA 468 breast cancer cells: mitogen-activated protein kinase (MAPK or ERK 1 and 2) pathways play a major role in early stages to cell migration; and protein kinase C δ isoforms (PKC-δ) play a significant role in later stages of sustained cell migration. Inhibition of MAPK activity with MAP kinase kinase (MEK) inhibitor PD98059 blocks early stages of cell migration (up to 4 h); however, cells revert back to enhanced cell migration after 4 h. While inhibition of PKC-δ activity with rottlerin or dominant-negative PKC-δ expression blocks sustained cell migration after 4 h and up to 12 h, the combination of MAPK and PKC inhibitors completely blocked transforming growth factor α (TGF-α)-induced cell migration in EGFR-overexpressing breast cancer cells. However, inhibition of MAPK activity completely blocked cell migration in low EGFR-expressing non-invasive breast cancer cells such as MCF-7 cells. Forced overexpression of EGFR in MCF-7 cells (EGFR/MCF-7 cells) resulted in cell migration patterns seen in MDA 468 cells, that is, MAPK pathways play a major role in early stages to cell migration, and PKC-δ plays a major role in later stages of sustained cell migration. The above data demonstrate that EGFR-overexpressing invasive cells have the ability to compensate the loss of MAPK-mediated signaling through activation of PKC-δ signaling for cell migration, which plays a major role in invasion and metastasis. In addition, data suggest that inhibition of MAPK and PKC-δ signaling pathways should abrogate cell migration and invasion in EGFR-overexpressing human breast cancer cells.

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Introduction

Cell migration is a fundamental process required during normal embryonic development, wound repair, inflammatory response, and tumor cell metastasis (1). There is abundant evidence to show that receptor tyrosine kinases are involved in development and progression of tumors. Expression and activity of epidermal growth factor receptor (EGFR) has been linked with cell migration, invasion, and metastasis (2–6). The binding of EGF or transforming growth factor α (TGF-α) to extracellular domain of EGFR activates its cytoplasmic tyrosine kinase, which undergoes autophosphorylation and recruits downstream effectors including PI3-K, Ras-Raf-mitogen-activated protein kinase (MAPK; ERK 1 and 2), and protein kinase C (PKC) signaling pathways, among others (7–9). All of these pathways have been shown to be involved in the regulation of EGFR-mediated cell proliferation, protease secretion, and cell migration (2–4, 10). In addition, it has been shown that cell lines that express high EGFRs exhibited reduced adhesion to matrix proteins when activated (10). These data suggest that EGFR-mediated signaling plays a major role in cell migration, and invasion, besides its well-known growth stimulatory activity.

The EGFR family of receptor protein tyrosine kinases consists of four known proteins: HER1 (also known as EGFR or ErbB1); HER2 (p185Neu or ErbB2); HER3 (ErbB3); and HER4 (ErbB4; 11, 12). Members of this subfamily have been implicated in various malignancies. HER receptors are present on many normal and tumor cells (13–15). Increased levels and/or amplification of EGFR have been found in many human tumors and cell lines, including breast cancer (16), gliomas (17), colon cancer (18), bladder cancer (19), tumors of the female genital tract (20), etc. Thus, it can be seen that EGFR expression occurs in many common tumors, and its overexpression is associated with invasion and metastasis in many patients. Understanding EGFR-mediated regulation of cell migration may provide novel approaches to control invasion and metastasis.

In the present study, we have identified a novel mechanism by which breast cancer cells that overexpress EGFR can circumvent the dependence of MAPK (ERK 1 and 2) for maintaining sustained cell migration. MCF-7 cells, which express low levels of EGFR (21), demonstrated only transient migratory ability on stimulation with TGF-α. In comparison, invasive and metastatic breast cancer cell lines, such as MDA 468 cells that express high EGFR (21), showed enhanced and sustained cell migration on stimulation with TGF-α. Inhibition of MAPK activity abrogates MCF-7 cells' transient motility, but has little effect on sustained cell migration in MDA 468 cells. In

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addition, we have shown that inhibition of MAPK activity reduced cell migration up to 4 h and then the cell continued to migrate at a rate similar to cells treated with TGF-α in MDA 468 and EGFR/MCF-7 cell lines. This suggests that EGFR overexpression is sufficient to induce a phenotype that is capable of inducing sustained cell motility independent of MAPK pathway. In addition, we have shown that specific inhibition of PKC-δ by rottlerin (a relatively specific PKC-δ inhibitor) or expression of dominant-negative PKC-δ was sufficient to abrogate the EGFR-mediated sustained cell migration. However, inhibition of calcium-dependent PKC isoforms with Go6976 had no effect on TGF-α-induced cell migration, suggesting that PKC-δ isoforms play a major role in EGFR-mediated, sustained cell migration. Similarly, inhibition of Rho-associated kinase (ROCK) activity blocks sustained cell migration in EGFR-expressing cells. The above data suggest that two distinct pathways may regulate cell migration in EGFR-overexpressing cells: one involves MAPK and myosin light chain kinase (MLCK) pathways in early stages to cell migration; whereas the other involves PKC-δ and ROCK pathways in sustained cell migration.

Results

High EGFR-Expressing Cell Lines Modulate Sustained Cell Migration Independent of MAPK but Dependent on PKC

MCF-7 breast cancer cells, which express low levels of EGFR (21), migrate on treatment with 100 ng/ml TGF-α (Fig. 1A). TGF-α was present for the entire duration of the experiments. Cell migration assays are done in triplicates and data presented in the figure(s) are means of at least three independent experiments. By 4 h, a 10-fold increase in the number of migratory cells was observed. However, this migration was only transient and after 4 h, no significant

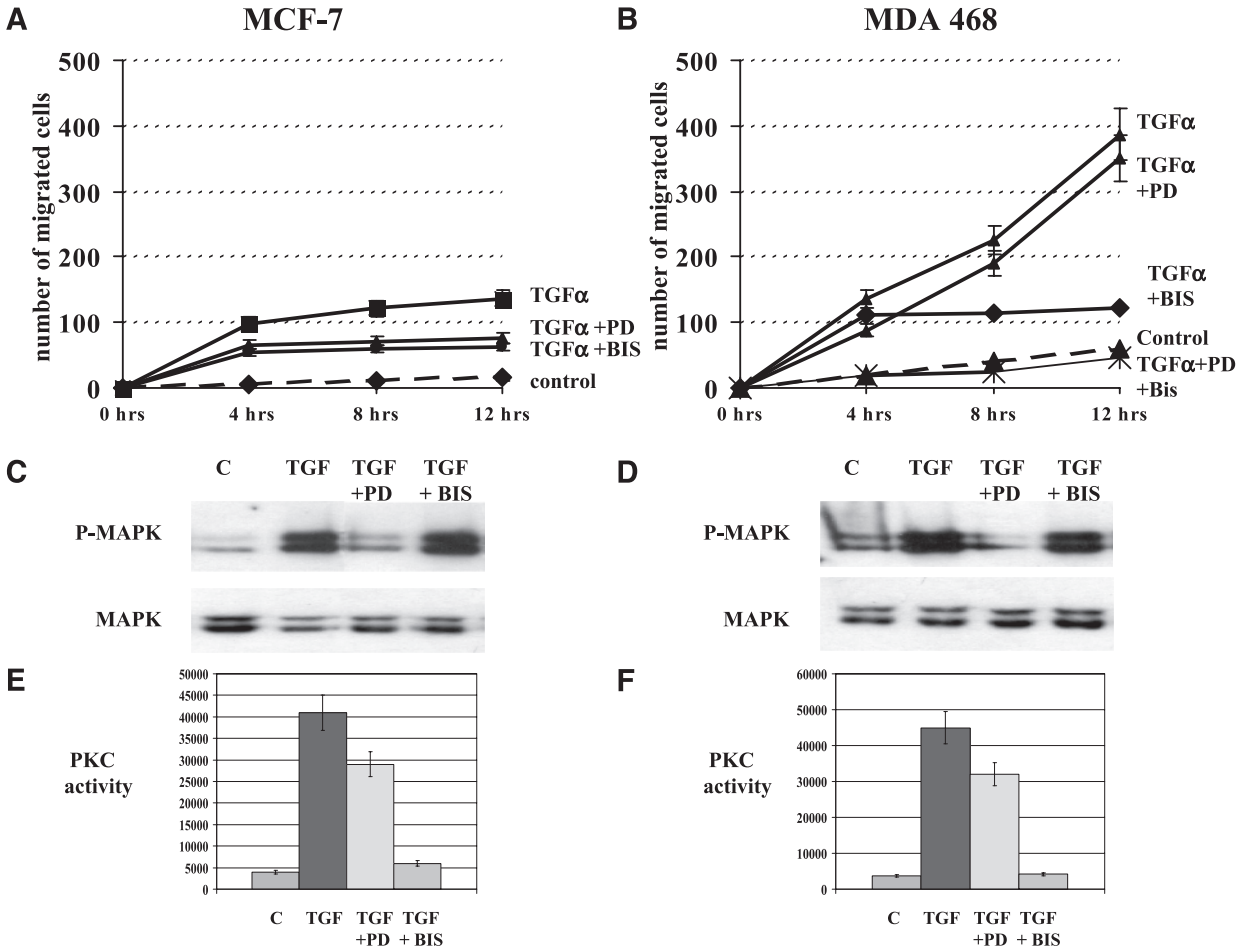

FIGURE 1. High EGFR-expressing cells have sustained cell migration. **A.** MCF-7 breast cancer cells migrate transiently with TGF-α stimulation, in contrast to MDA 468 cells. **B** demonstrated sustained migration with TGF-α treatment. Inhibition of MAPK activation using PD98059 (*PD*) blocks transient (up to 4 h) migration in both cell lines, but it was unable to block the sustained cell migration seen in MDA 468 cells (**A**, **B**). However, inhibition of PKC activity using bisindoylmaleimide (*Bis*) was sufficient to block the sustained migration observed in MDA 468 cells (**B**). Inhibition of MAPK and PKC activity almost completely blocks cell migration. **C** and **D** show that MAPK activity is blocked by MEK inhibitor PD98059 but PKC inhibitor Bis had no effect on TGFα-induced MAPK activity in both MCF-7 and MDA 468 cells. PKC inhibitor Bis blocks the activity of most PKC isoforms (**E** and **F**), without significantly affecting TGF-α-induced MAPK activity. Cell migration assays are done in triplicates and data are means of at least three independent experiments.

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increase in additional migratory cells was observed (Fig. 1A). However, when cells are treated with MAP kinase kinase (MEK) inhibitor PD98059 (40 μM), TGF-α-induced MAPK activity was significantly reduced (Fig. 1C) and this led to reduced MCF-7 cell migration (47%, $P < 0.05$) as observed at 4 h and the abrogation of cell motility thereafter (Fig. 1A). This demonstrates the requirement for MAPK activity in regulating cell migration in low EGFR-expressing, non-invasive MCF-7 cells.

In contrast, the high EGFR-expressing cell line MDA 468 demonstrates sustained cell motility with 100 ng/ml TGF-α stimulation throughout the time course observed (Fig. 1B). Blockade of TGF-α-induced MAPK activity (Fig. 1D) with 40 μM PD98059 only showed modest inhibition of cell motility (35% by 4 h, $P < 0.05$; Fig. 1B). More significantly, after the initial 4 h, cells treated with PD98059 continue to migrate at a rate similar to cells treated with TGF-α (Fig. 1B). Western blot analysis demonstrated that MAPK activity was inhibited by treatment with MEK inhibitor PD98059 up to 12 h (Fig. 1D). This indicates that MAPK activation may play a major role in early stages of cell migration (up to 4 h); however, it may not be required for sustained phase of cell migration in high EGFR-expressing cells. However, the sustained cell motility normally observed after 4 h in these cells was significantly reduced by using the broad-spectrum PKC inhibitor such as Bis (5 μM; Fig. 1B). PKC inhibitor Bis blocks the activity of most of the PKC isoforms (Fig. 1, E and F) without significantly affecting TGF-α-induced MAPK activity (Fig. 1, C and D). These data suggest that there is discrete and differential regulation of the transient and sustained phases of TGF-α-induced cell migration in high EGFR-expressing cells regulated by both MAPK and PKC, respectively.

EGFR Overexpression Induces Sustained Cell Motility

To determine if sustained cell motility was specifically dependent on high EGFR numbers, we created EGFR-overexpressing MCF-7 cells (EGFR/MCF-7). In vector control MCF-7 cells, EGFR and its phosphorylation was not readily detectable due to low EGFR number on these cells. In contrast, EGFR/MCF-7 clones (#4 and #14) showed significantly higher EGFR phosphorylation due to the high EGFR expression (11- and 28-fold, respectively) in the presence of TGF-α (Fig. 2B). EGFR/MCF-7 cells and vector control cells showed similar cell migration for up to 4 h in the presence of

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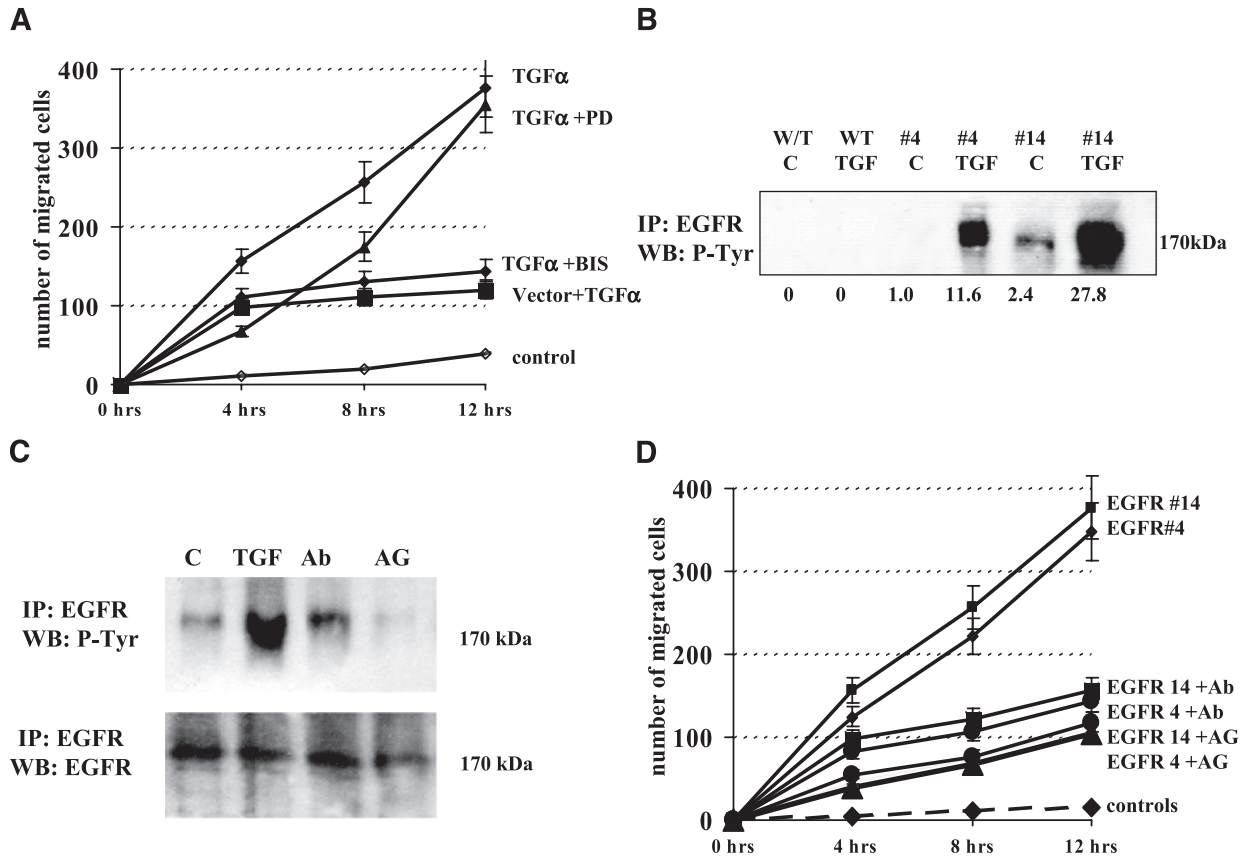

FIGURE 2. Sustained migration is dependent on EGFR-mediated signaling. Cell migration in the EGFR-overexpressing MCF-7 (EGFR/MCF-7) clones was sustained over the time course, and this resulted in a 3-fold increase over vector control MCF-7 cells (**A**) and this correlates enhanced EGFR phosphorylation (**B**). The use of EGFR blocking antibody (*Ab*) or tyrosine kinase inhibitor AG1478 (*AG*) resulted in reduced EGFR phosphorylation (**C**) and significant reduction in sustained migration of the EGFR/MCF-7 clones (**D**), demonstrating the dependence on EGFR-mediated signaling for sustained cell migration.

TGF-α (Fig. 2A). However, after this transient induction of cell migration, vector control cells migrate only at basal levels throughout the 12-h time course (Fig. 1A). In contrast, the EGFR/MCF-7 clones (#4 and #14) show sustained cell migration throughout the 12-h time course, resulting in a 3-fold increase in overall cell motility compared to vector control MCF-7 cells by 12 h in the presence of TGF-α ($P < 0.05$; Fig. 2A). These data support the concept that EGFR overexpression can lead to a more aggressive phenotype as demonstrated by the enhanced and sustained cell migration.

To further confirm that sustained cell motility is dependent on EGFR-mediated cell signaling, we blocked EGFR-mediated signaling with EGFR antibody (Ab 225), which was previously shown by our group and others (22, 23) to be specific for EGFR. Addition of EGFR antibody significantly reduces TGF-α-induced receptor phosphorylation in EGFR/MCF-7 cells (data for clone 14 are shown; Fig. 2C). This reduced EGFR phosphorylation correlated with significant reduction of cell migration at 4 h ($P < 0.05$; Fig. 2D). To further confirm the role of EGFR signaling in cell migration, we used 10 μM AG1478, a tyrosine kinase inhibitor that has been shown to be effective in blocking EGFR activity *in vitro* (24, 25). AG1478 also significantly blocked the TGF-α-induced EGFR phosphorylation and cell motility throughout the time course ($P < 0.05$; Fig. 2, C and D). This correlated with an average 66% reduction in cell motility observed throughout the time course ($P < 0.05$; Fig. 2D). These data suggest that EGFR-mediated signaling plays a major role in sustained migration observed in these cells.

PKC-δ Plays a Major Role in Sustained EGFR-Mediated Cell Motility

Specific PKC isoforms have been shown to play important roles in breast cancer cell migration (26, 27). Our data show that broad-spectrum PKC inhibitor Bis abrogated sustained cell migration in both the EGFR/MCF-7 clones and MDA 468 cell lines after 4 h up to 12 h (Figs. 1B and 3, A and B). To identify specific PKC isoforms that may regulate EGFR-induced sustained cell motility, we used Go6976, an inhibitor of the classical, calcium-dependent isoforms (PKC-α, -β, -γ; 28), and rottlerin, a relatively specific inhibitor of PKC-δ (27, 29). Rottlerin was able to significantly reduce (70% by 12 h; $P < 0.05$) the TGF-α-induced sustained cell migration observed in the high EGFR-expressing EGFR/MCF-7 clones and MDA 468 cells (Fig. 4, A and B). In contrast, inhibition of Ca^{2+}-dependent PKC activity by Go6976 did not affect cell motility (Fig. 3, A and B). These data suggest that PKC-δ isoform plays a major role in EGFR-mediated cell migration.

To further confirm the role of PKC-δ in sustained cell migration, a dominant-negative PKC-δ (30) that has a mutation in the ATP binding site was transiently transfected into MCF-7 control or EGFR/MCF-7 cells. Transient transfection showed a 70–80% decrease in PKC-δ activity in the control and EGFR cells (Fig. 4B). This resulted in significant reduction of sustained cell migration in EGFR/MCF-7 clones (Fig. 4A). In contrast, the transient cell migration (up to 4 h) in MCF-7 parental or EGFR/MCF-7 clones transfected with dominant-negative PKC-δ was not altered significantly (Fig. 4A). These data are consistent with our hypothesis that early stages (up to 4 h) of cell migration are mediated through MAPK signaling pathway and persistent (from 4 h up to 12 h) cell migration is mediated by PKC-δ-mediated signaling pathway.

Our data suggest that EGFR-induced sustained cell motility is regulated by Rho but not MLCK in a PKC-δ-mediated pathway. Both MLCK and ROCK were shown to play a critical role in modulating cell migration (13, 31, 32). In addition, it was previously shown that both MLCK and ROCK have the ability to phosphorylate MLC by distinct signaling pathways (31–34). To determine if PKC-δ signaling modulates either of these pathways, we blocked MLCK activity by inhibitor ML-7 and ROCK activity by a relatively specific ROCK inhibitor Y27632 (31–35). The EGFR clones show higher basal levels and nearly a 2-fold increase in PKC-δ activity compared to vector control cells (Fig. 5C). None of the inhibitors used reduced PKC-δ activity significantly. Inhibition of MLCK activity by ML-7 inhibitor blocked cell migration in MCF-7 vector control in a manner similar to that seen with MEK inhibitor PD98059 (Fig. 5A). MLCK inhibitor ML-7 only blocked early stages of cell migration (up to 4 h), but had no effect on sustained cell migration in the EGFR/MCF-7 clones (Fig. 5B). This indicates that the MLCK signaling pathway plays a major role in early stages of cell migration, but not in the sustained motility observed in the EGFR-overexpressing cells.

Alternatively, the use of the ROCK inhibitor Y27632 had a significant effect on the sustained cell migration in the EGFR-overexpressing cells (Fig. 5B). Addition of Y27632 to EGFR-overexpressing cells significantly reduces the sustained cell migration in a pattern similar to those seen with PKC-δ inhibitor rottlerin (Figs. 3B and 5B) or dominant-negative PKC-δ (Fig. 4A). This demonstrates that EGFR-induced sustained cell motility is dependent on ROCK, but independent of MLCK signaling. Most significantly, these data provide evidence that ROCK and PKC-δ are both involved in regulation of sustained cell motility. To further demonstrate that the Rho-mediated signaling plays a major role in sustained cell migration, we also used dominant-negative N19 Rho that has been shown to significantly block ROCK activation. Transfection of EGFR/MCF-7 cells with dominant-negative N19 Rho abrogated sustained cell migration (Fig. 6). The above data suggest that PKC-δ- and ROCK-mediated signaling plays a major role in sustained cell migration in EGFR-overexpressing breast cancer cells.

Discussion

Earlier publications from our group and others have shown that activation of the MAPK (ERK 1 and 2) signaling pathway plays a major role in growth factor-induced cell migration (35, 36). Mechanistically, this was shown to be accomplished through phosphorylation of MLCs by MLCK as a result of phosphorylation on MLCK by MAPK (35, 37). Our results agree with the previous studies that provide a role for MAPK signaling in modulating cell migration; however, in this study, we have shown that two distinct pathways regulate cell migration in EGFR-overexpressing cells: early stages of cell migration are dependent on MAPK through the activation of

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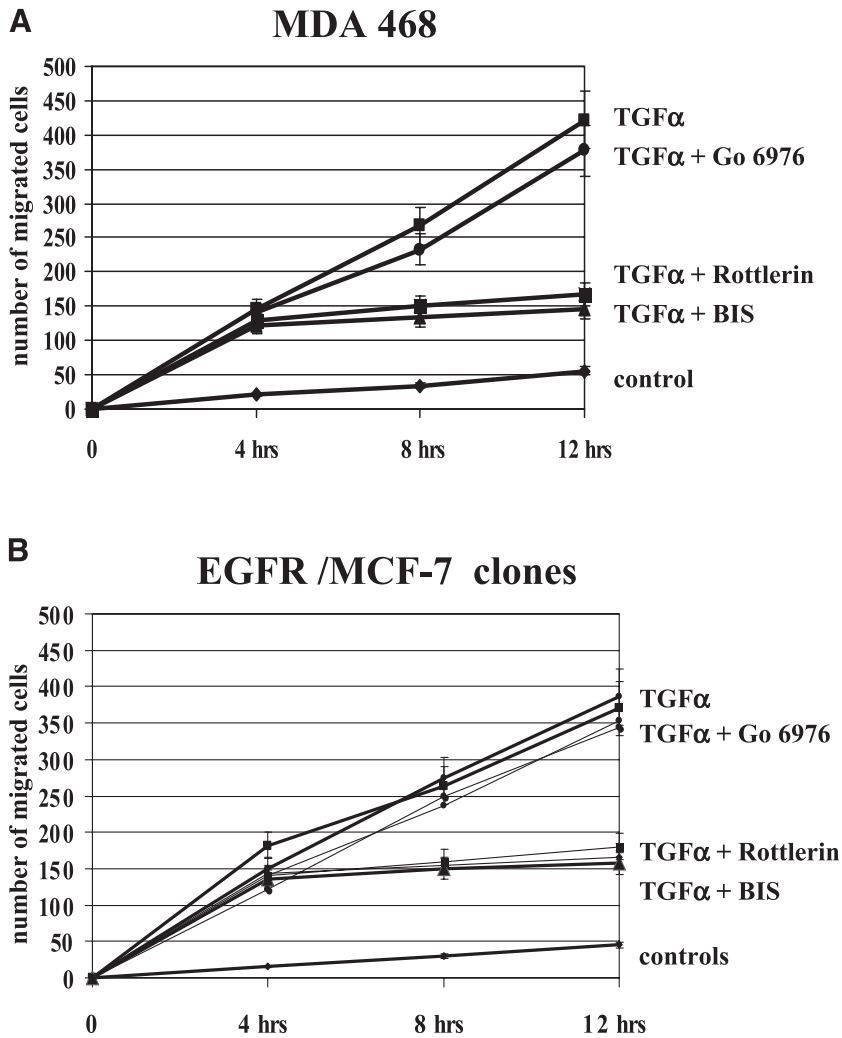

FIGURE 3. The specific PKC isoform PKC-δ blocks sustained cell migration. Inhibition of PKC activity by broad-spectrum PKC inhibitor Bis or PKC-δ specific inhibitor rottlerin had little effect on transient cell migration (up to 4 h), but these inhibitors significantly abrogated sustained cell migration in both MDA 468 and EGFR/MCF-7 cells (from 4 h up to 12 h; **A**, **B**). Inhibition of PKC-δ by rottlerin significantly blocked TGF-α-induced cell migration, but inhibition of Ca^{2+}-dependent PKC activity by Go6976 had marginal effect on cell migration (**A**, **B**). These data suggest that PKC-δ plays a major role in TGF-α-induced sustained cell migration in EGFR-overexpressing cells.

MAPK/MLCK signaling mechanism; and later stages of cell migration are dependent on the specific PKC isoform PKC-δ- and ROCK-mediated pathways. Our data demonstrated that EGFR-overexpressing cell lines such as MDA 468 and EGFR/MCF-7 have the ability to maintain sustained cell migration despite inhibition of MAPK activity by MEK inhibitor PD98059. However, inhibition of PKC-δ by rottlerin or expression of dominant-negative PKC-δ abrogates this sustained cell migration, suggesting that MAPK-independent but PKC-δ-dependent mechanism modulates sustained cell migration in EGFR-overexpressing cells. This observation may provide an explanation for the invasive and metastatic phenotype that is seen in EGFR-overexpressing cancer cells. Although MAPK signaling is a significant regulator of cell migration in a wide variety of cell types, EGFR overexpression can circumvent the dependence on MAPK for sustained cell migration. Activation of PKC-δ by EGFR can introduce a novel PKC-δ-dependent mechanism that regulates existing or novel downstream signaling mechanisms that are involved in the regulation of cell migration. Howe and Juliano (38) reported a similar mechanism for integrin-mediated activation of the Raf/MEK/MAPK cascade, which comprises of initial PKC-independent phase that is optimized by translocation of Raf to plasma membrane, followed by a sustained phase that is blocked by PKC inhibitors. Recent data from another group show that in MDA 231 cells, inhibition of MAPK activity by PD98059 only blocked basal unstimulated migration, but it had no effect on EGF-stimulated migration (39), suggesting that EGFR overexpression regulates cell migration independent of MAPK signaling pathway.

It is well documented that PKC levels are elevated in malignant tumors when compared to normal breast tissue (40–42). In this study, we have shown that increased PKC-δ isoform could contribute to invasion and metastatic process by increasing cell migration. The involvement of PKC and ROCK in regulation of cell migration has been demonstrated previously (43–45). The mechanism by which PKC-δ regulates sustained cell migration is not fully understood. However, one of the possible mechanisms may be that PKC regulates cell migration through the activation of Src and FAK and subsequent establishment of Cas-Crk complex for sustained cell migration was shown to be PKC dependent (44). Other studies have implicated PKC-δ in the regulation of cellular processes important for tumor progression (27, 43, 46).

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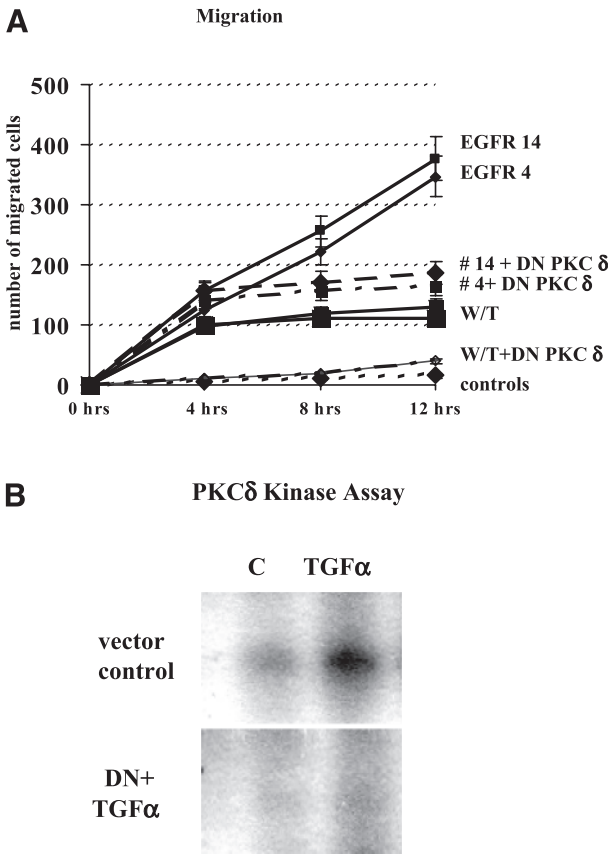

FIGURE 4. Dominant-negative PKC-δ blocks sustained migration. Transfection of dominant-negative PKC-δ (*DN*) into EGFR/MCF-7 clones abrogated most of the PKC-δ activity in the cells as determined by kinase assay (**B**). This resulted in a significant reduction in sustained cell migration (**A**), suggesting that PKC-δ plays a major role in cell migration of EGFR-overexpressing cells.

Depletion of PKC-δ by TPA results in abrogation of migration in colonic epithelial cells, indicating a requirement for the specific isoenzyme PKC-δ during cytoskeletal rearrangement (43). Another study has shown that overexpression of PKC-δ had no effect on growth of the cells, but it regulated anchorage-independent growth and lung colony formation of breast cancer cells (45). In addition, it has been shown that Rho acts via ROCKs to affect MLC phosphorylation, both by inhibition of MLC phosphatase and by phosphorylating MLC (47, 48). MLC phosphorylation is also regulated by MLCK, which is stimulated by the MAPK (35). Our data show that inhibition of MLCK activity only blocked early stages of cell migration (Fig. 5), but it had no marginal effect on sustained cell migration, suggesting that ROCK mediates cell migration independent of MLCK and MLC in EGFR-overexpressing cells. It is likely that MLCK, PKC-δ, and ROCK act in concert to regulate different aspects of cell migration in EGFR-overexpressing cells. However, at present, it is not clear whether PKC-δ acts through ROCK or both independently mediate sustained cell migration. It was previously shown that activation of the Rho-ROCK pathway may be one of the major steps during the transcellular migration using rat MM1 hepatoma cells (49). The involvement of all these signaling molecules is not surprising given the diversity of extracellular signals that affect cell migration, and the number of cellular responses that have to be coordinated such as lamellipodium extension, formation of new adhesions, cell body contraction, and tail detachment.

In this study, we have identified a novel mechanism by which cancer cells that overexpress EGFR can circumvent the dependence on MAPK for maintaining sustained cell migration. Our data suggest that two distinct pathways regulate cell migration in EGFR-overexpressing cells such as MDA 468 or EGFR/MCF-7 breast cancer cells: one involves MAPK in early stages to cell migration; the other involves PKC-δ, which regulates persistent cell migration independent of MAPK. The antagonist of MAPK (PD98059) or MLCK which blocks MLC phosphorylation blocks early cell migration (up to 4 h); however, cells revert back to enhanced cell migration after 4 h. While inhibition of PKC-δ activity or ROCK with relatively specific inhibitors or dominant-negative expression blocks cell migration after 4 h and up to 12 h, the combination of MAPK and PKC-δ inhibitors completely blocked TGF-α-induced cell migration in EGFR-overexpressing cells on extracellular matrix. Thus, this study provides significant insight into the contribution of EGFR signaling to regulation of sustained cell motility and also provides a new target to block growth factor-stimulated cell migration in general.

Materials and Methods

Cell Lines and Reagents

MCF-7 and MDA 468 cells were obtained from American Type Culture Collection (Rockville, MD) and culture conditions have been described briefly. All breast cancer cells were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD), supplemented with 5% FCS (Life Technologies) and insulin (Life Technologies) for MCF-7 cells and 10% FCS for MDA cell lines. All cell lines were routinely tested for mycoplasma contamination and found to be negative. TGF-α was purchased from R&D Systems, Inc. (Minneapolis, MN) and used at a final concentration of 100 ng/ml. PD98059 was obtained from Calbiochem (San Diego, CA), dissolved in DMSO, and used in a final concentration of 40 μM. Bis was obtained from Calbiochem, dissolved in DMSO, and used in a final concentration of 5 μM. Rottlerin was obtained from Calbiochem, dissolved in DMSO, and used in a final concentration of 5 μM. Go6976 was obtained from Calbiochem, dissolved in DMSO, and used in a final concentration of 1 μM. AG1478 (EGFR inhibitor) and Y27632 (ROCK inhibitor) were obtained from Calbiochem and dissolved in DMSO. The final concentration of DMSO did not exceed 0.1% in any experiment. Antibodies for MAPK, ERK1, and ERK2 were obtained from Zymed Laboratories (San Francisco, CA). Phospho-MAPK was obtained from Cell Signaling Technologies (Beverly, MA). Anti-EGFR antibody (Ab 225) for inhibition experiments was obtained from Oncogene (San Diego, CA). EGFR antibody used for Western blotting was EGFR Cocktail (Ab-12) from Neomarkers (Fremont, CA). EGFR antibody used for immunoprecipitation was EGFR 1005 from Santa Cruz Biotechnology (Santa Cruz, CA).

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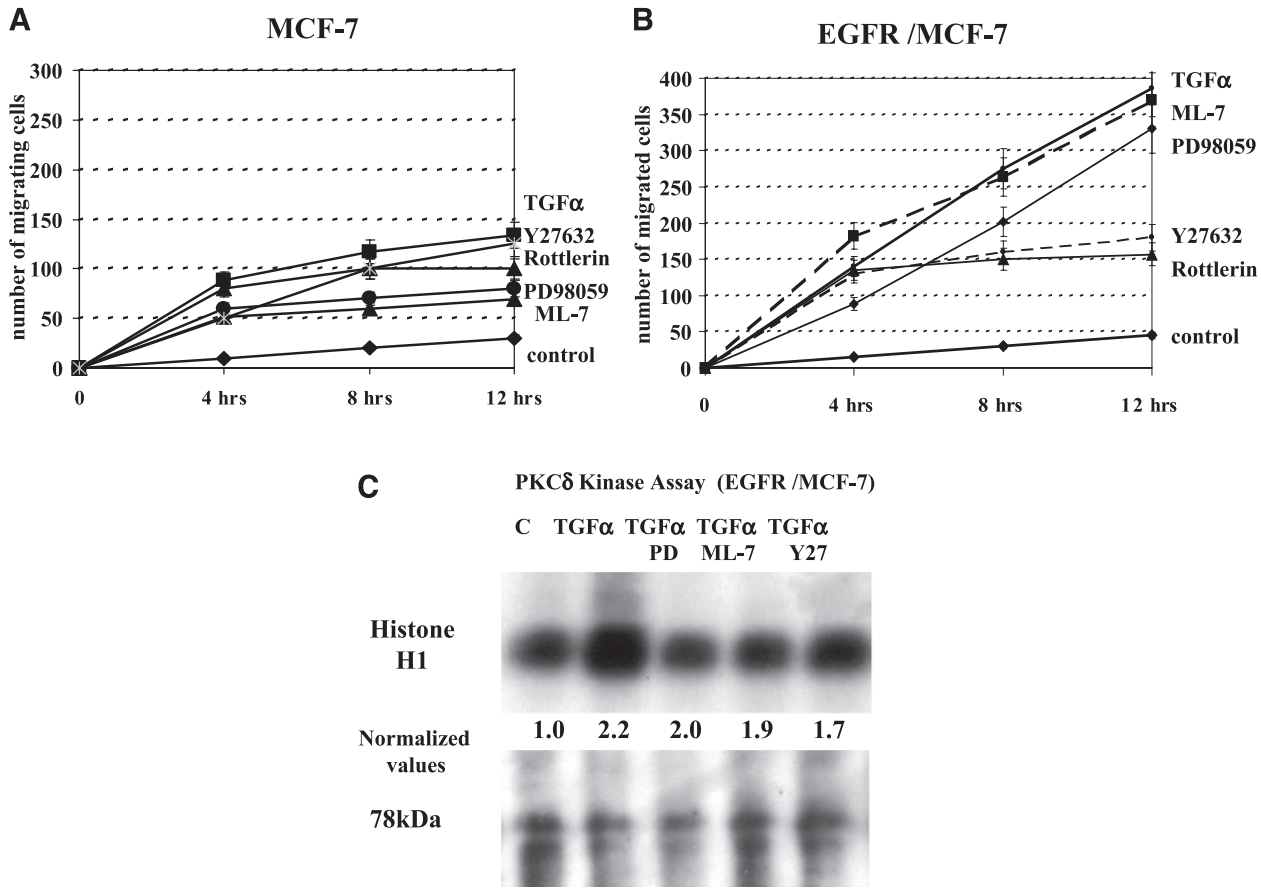

FIGURE 5. ROCK inhibition but not MLCK inhibition is sufficient to block sustained cell motility in EGFR/MCF-7 cells. The use the MLCK inhibitor ML-7 significantly reduced MCF-7 transient cell motility (**A**, **B**), but had no effect on sustained cell motility in the EGFR/MCF-7 clones. **B.** In contrast, ROCK inhibition, using Y27632, was sufficient to block sustained cell motility in EGFR/MCF-7 cells (**B**). This type of inhibition correlated with the effects of PKC-δ inhibition using rottlerin (**B**) despite no significant effects of these inhibitors of PKC-δ activity (*bottom panels*). Thus, we propose that sustained cell motility relies on ROCK in a PKC-δ-mediated mechanism.

HRP-conjugated phospho-tyrosine (PY-20) was obtained from Transduction Laboratories (San Diego, CA). PKC-δ antibody for immunoprecipitation (C-20) was from Santa Cruz Biotechnology. PKC-δ antibody for Western blotting (P36520) was from Transduction Laboratories.

Plasmid Constructs and Transfection

EGFR cDNA was obtained from Dr. Gordon Gill, Department of Endocrinology and Metabolism, University of California in San Diego and placed in the pcDNA3 mammalian expression vector. Clones were initially screened for EGFR expression and phosphorylation. HA-tagged wild-type or dominant-negative PKC-δ (consisting of K to R mutation in the ATP binding site) was obtained from Dr. Bernard Weinstein, Herbert Irving Comprehensive Cancer Center, Columbia University, and cloned into pcDNA3. All stable clones were made by electroporation (220 V, 0.1 ms, 960 μF) of 100,000 cells with 1 μg of DNA in 1 ml. Cells were seeded on 100-mm plates, allowed to grow in regular growth medium for 1 week, and then selected in selection media (250–1000 μg/ml G418) for 2–3 weeks before isolating colonies. Approximately 50–100 individual colonies were isolated and grown to confluency in 24-well dishes, transferred to 6-well dishes, and grown in selection media for 2–3 weeks. Surviving clones were screened for protein expression, then functionality. Transient transfections were performed by using Superfect Transfection Reagent (Qiagen, Valencia, CA) as directed. Two micrograms of plasmid DNA were transfected into a 30-mm dish, cells allowed to recover 72 h, and serum starved 24 h before the beginning of each experiment.

Western Immunoblot Analysis

Western blotting was performed as described previously using a standard protocol (36). Crude protein extracts were obtained by lysing 5×10^6 cells in a buffer [50 mM Tris-HCl (pH 7.6), 1% NP40, 2 mM EDTA, 0.5% Na deoxycholate, 150 mM NaCl, 1 mM Na orthovanadate, 2 mM EGTA, 4 mM Na *p*-nitrophenylphosphate, 100 mM Na fluoride] supplemented with protease inhibitors [leupeptin (0.5%), aprotinin (0.5%), and phenylmethylsulfonyl fluoride (0.02%)]. Samples containing 50 μg of total protein were electrophoresed on 7.5% (for EGFR) and 10% (for MAPK) SDS-polyacrylamide gels and

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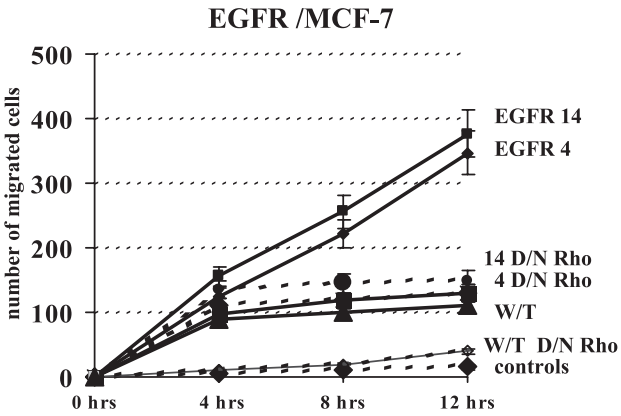

FIGURE 6. Dominant-negative Rho blocks sustained cell motility. EGFR/MCF-7 cells transfected with N19 Rho lose the ability to maintain sustained cell motility, further emphasizing the requirement of ROCK signaling.

transferred onto nitrocellulose membranes by electroblotting. Membranes were probed with antibodies as indicated, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham, Piscataway, NJ) and enhanced chemiluminescence detection (Amersham). For quantification of activity, band intensities of the phospho- and total protein(s) were quantified using Bio-Rad's "Quantity one" software (Bio-Rad, Hercules, CA).

Migration Assays

Cells are grown to 60% confluency on six-well plastic dishes and serum starved for 24 h. Cells are washed with PBS and detached with 500 μl of 1 mM EDTA/0.25% trypsin. Cells are resuspended to an appropriate volume of serum-free/phenol red-free media with 0.25% soybean trypsin inhibitor (Life Technologies) giving 100,000 cells per milliliter final concentration. The drug was added to the suspended cells and incubated for 30 min. Then, growth factor was added to the cells and the cells plated onto a 24-well 8 μM polycarbonate filter assay system (Falcon Transwell, Becton Dickinson, Franklin Lakes, NJ) that has the backside of the filter in each well coated with 30 μl of 10 μg/ml rat tail collagen I (BD Biosciences, San Diego, CA). The cells are allowed to migrate through the pores and attach to the backside for a determined amount of time, then they are fixed to the backside with HEMA 3 (Fisher Scientific, Hanover Park, IL) fixative. The topside was washed and the cells scraped off, then the cells are stained with HEMA 3 staining kit (Fisher) giving the cells a blue color (eosin Y/methylene blue differential staining kit). The cells can now be visualized and counted under a microscope.

Total PKC and PKC-δ Phosphorylation Assay

Cells were grown to 50–60% confluency, serum starved in phenol red-free DMEM with 0.1% BSA for 24 h, and pretreated with drug or DMSO vehicle alone for 30 min before adding 100 ng/ml TGF-α. Cells were lysed as described in Western blotting protocol. Alternatively, cells were transfected with 5 μg plasmid as described in transient transfection protocol and treated as described above. Immunoprecipitation of 500 ng of protein using 2 μg of anti-PKC-δ antibody (Santa Cruz Biotechnology sc-937) followed by Protein G-plus agarose beads (Calbiochem) were performed as described earlier (36) and washed 3× with 1 ml PBS. Forty-five microliters of assay reaction mixture {10 μM phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO) and 0.3 mg/ml phosphatidylserine (Sigma), 20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM ATP, 100 μM ([γ-^{32}P]ATP (100 mCi/mmol)), with 0.2 mg/ml histone H1 as the substrate} were added to the beads, mixed, and incubated at 30°C for 5 min. Reaction was terminated by adding 5 μl of 100 mM EDTA. Samples were boiled for 5 min, centrifuged briefly, and 25 μl of supernatant loaded on a 12% SDS-PAGE gel. The gel was transferred onto a nitrocellulose membrane and exposed to X-ray film for 4 h to overnight. The corresponding autoradiograph shows the activity of PKC-δ to phosphorylate substrate at a given time period under a given condition. Total PKC activity was done by PKC assay kit (Calbiochem) according to the manufacturer's protocol.

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