Protein Kinase C α Mediates Erlotinib Resistance in Lung Cancer Cells

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ABSTRACT

Overexpression and mutational activation of the epidermal growth factor receptor (EGFR) plays an important role in the pathogenesis of non-small cell lung cancer (NSCLC). EGFR tyrosine-kinase inhibitors (TKIs) are given as a primary therapy for advanced patients with EGFR-activating mutations; however, the majority of these tumors relapse and patients eventually develop resistance to TKIs. To address a potential role of protein kinase C (PKC) isozymes in the resistance to TKIs, we used the isogenic NSCLC H1650 cell line and its erlotinib-resistant derivative H1650-M3, a cell line that displays a mesenchymal-like morphology driven by transforming growth factor- β signaling. We found that H1650-M3 cells display remarkable PKC α upregulation and PKC δ downregulation. Notably, silencing PKC α from H1650-M3 cells using RNA interference caused

a significant reduction in the expression of epithelial-to-mesenchymal transition (EMT) markers vimentin, Zeb2, Snail, and Twist. Moreover, pharmacological inhibition or PKC α RNA interference depletion and PKC δ restoring sensitized H1650-M3 cells to erlotinib. Whereas ectopic overexpression of PKC α in parental H1650 cells was not sufficient to alter the expression of EMT genes or to confer resistance to erlotinib, it caused downregulation of PKC δ expression, suggesting a unidirectional crosstalk. Finally, mechanistic studies revealed that PKC α upregulation in H1650-M3 cells is driven by transforming growth factor- β . Our results identified important roles for specific PKC isozymes in erlotinib resistance and EMT in lung cancer cells, and highlight PKC α as a potential target for lung cancer treatment.

Introduction

Lung cancer remains one of the major causes of mortality worldwide, accounting for more deaths than any other cancer (Kanne, 2014; Ferlay et al., 2015). Diagnosis of lung cancer normally occurs in late stages of the disease, thus limiting the options for treatment. The most common type of lung cancer (approximately 85%) is non-small cell lung cancer (NSCLC), which has three main types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (Molina et al., 2008; Shames and Wistuba, 2014). Genetic alterations in NSCLC tumors primarily include oncogenic mutations in the epidermal growth factor receptor (*EGFR*) and *KRAS*, as well as inactivation of tumor suppressor genes such as *p53*, *PTEN*, *Rb*, and *p16* (Hollstein et al., 1991; Reissmann et al., 1993; Jin et al., 2010). Mutations in the *EGFR* gene, particularly deletion of exon 19 and L858R mutation in exon 21, occur in 10–50% of NSCLC patients (Gazdar, 2009; Cooper et al., 2013). Small molecule tyrosine-kinase inhibitors (TKIs) that

reversibly inhibit EGFR at the ATP pocket domain, such as erlotinib and gefitinib, currently represent the first line of therapy for EGFR-mutated NSCLC patients (Antonicelli et al., 2013; Steins et al., 2014). Although these therapies are initially efficacious, ultimately most patients develop resistance. Whereas resistance has been attributed in some cases to the acquisition of secondary EGFR mutations or MET amplification (Kobayashi et al., 2005; Engelman et al., 2007), the mechanisms behind the resistance to TKIs are only partially understood. Dissecting the signaling mechanisms driving resistance is crucial for designing combinational therapy regimes to overcome this hurdle and extend life expectancy of NSCLC patients.

Protein kinase C (PKC) represents a group of serine-threonine kinases involved in a variety of cellular functions, including mitogenesis, survival, and motility. The PKC family is composed of 10 members classified into three classes: calcium-dependent or conventional PKCs (cPKC α , cPKC β I, cPKC β II, and cPKC γ), calcium-independent or novel PKCs (nPKC δ , nPKC ϵ , nPKC η , and nPKC θ), and phorbol ester/diacylglycerol unresponsive or atypical PKCs (aPKC ζ and aPKC ι/λ) (Barry and Kazanietz, 2001; Newton, 2001; Griner

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ABBREVIATIONS: AdV, adenovirus; aPKC, atypical protein kinase; cPKC, conventional protein kinase C; Ct, cycle threshold; EGFR, epidermal growth factor receptor; EMT, epithelial-to-mesenchymal transition; GF109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide; Gö6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; LY2109761, 4-[5,6-dihydro-2-(2-pyridinyl)-4H-pyrrolo[1,2-b]pyrazol-3-yl]-7-[2-(4-morpholinyl)ethoxy]-quinoline; MOI, multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; nPKC, novel protein kinase C; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PKC, protein kinase C; qPCR, quantitative polymerase chain reaction; RNAi, RNA interference; TKI, tyrosine-kinase inhibitor.

and Kazanietz, 2007; Garg et al., 2014; Parker et al., 2014). Decades of research have established key roles for different members of the PKC family in the progression of cancer. It became clear that individual PKC isozymes could act either as tumor promoters or tumor suppressors. For example, PKC β has been proposed to be involved in lung tumorigenesis, and the PKC β inhibitor enzastaurin has been examined as a potential therapeutic agent for lung cancer patients (Tekle et al., 2008; Willey et al., 2010; Vansteenkiste et al., 2012; El Osta et al., 2014). Our laboratory recently showed that PKC ϵ , a kinase implicated in cell cycle progression and motility, is required for the tumorigenic and metastatic activities of NSCLC cells (Caino et al., 2012a,b). On the other hand, PKC α and PKC δ negatively modulate NSCLC cell cycle progression (Nakagawa et al., 2005; Santiago-Walker et al., 2005; Oliva et al., 2008; Xiao et al., 2008). Most recently, Hill et al. (2014) provided direct evidence for a tumor suppressive role for PKC α in KRAS tumorigenesis. The fact that PKC α promotes NSCLC cell migration (Cheng et al., 2009; O'Neill et al., 2011) suggests divergent roles for this kinase in different stages of lung cancer progression. Likewise, diverse roles for PKC α and other members of the PKC family have been established in survival of NSCLC cells and other cancer cell types (Garg et al., 2014). In addition, the overexpression of some PKC family members has also been associated with low sensitivity to the irreversible TKI afatinib in lung cell line models (Coco et al., 2014).

Toward the goal of determining a potential involvement of PKC isozymes in TKI resistance in lung cancer, here we took advantage of an isogenic NSCLC cell model of erlotinib resistance generated by culturing the parental H1650 cell line in the presence of a high concentration of the inhibitor. Erlotinib-resistant H1650 cells display features of epithelial-to-mesenchymal transition (EMT), a phenotype that is maintained by the transforming growth factor- β (TGF- β) pathway (Yao et al., 2010). Our study identified discrete roles for PKC isozymes, specifically PKC α and PKC δ , in erlotinib resistance and EMT in NSCLC cells.

Materials and Methods

Reagents. Erlotinib hydrochloride was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pan-PKC inhibitor GF109203X (2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide) was purchased from Enzo Life Sciences (Plymouth Meeting, PA). The cPKC inhibitor Gö6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile) was obtained from LC Laboratories (Woburn, MA).

Cell Culture. The H1650-M3 cell line was derived from parental H1650 cells in the laboratory of Dr. Raffaella Sordella (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) (Yao et al., 2010). Both cell lines were kindly provided by Dr. Sordella, and were cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and maintained at 37°C in a humidified 5% CO $_2$ atmosphere.

Real-Time Polymerase Chain Reaction. Total RNA was extracted from subconfluent cell cultures using the RNeasy kit from Qiagen (Valencia, CA). Total RNA (1 μ g) was reverse transcribed to cDNA using the TaqMan reverse transcription reagent kit (Applied Biosystems, Branchburg, NJ). Real-time quantitative polymerase chain reaction (qPCR) was performed essentially as described (Wang et al., 2014) using an ABI PRISM 7700 detection system (Applied Biosystems). The reaction was carried out in triplicate samples containing TaqMan Universal PCR MasterMix (Applied Biosystems),

target primers (300 nM), fluorescent probe (200 nM), and 4 μ l transcribed cDNA (6 $\times$ dilution). TaqMan primers 5'-end labeled with 6-carboxyfluorescein for PKC α , PKC δ , E-cadherin, Snail, Twist1, vimentin, Zeb2, and 18S rRNA (housekeeping gene) were purchased from Applied Biosystems. PCR product amplification was continuously monitored using the sequence detection system software (version 1.7; Applied Biosystems). Triplicate cycle threshold (Ct) values were averaged and normalized to an average 18S Ct value to calculate the Δ Ct. The Δ (Δ Ct) was determined by subtracting the control Δ Ct value from the experimental Δ Ct value. Fold changes were expressed as $2^{-\Delta(\Delta Ct)}$.

Western Blot Analysis. Western blot analysis was carried out essentially as previously described (Oliva et al., 2008). Briefly, cells were harvested in lysis buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.08% bromophenol blue, and 5% β -mercaptoethanol). Samples were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MD). After blocking with 5% milk in 1% Tween 20/phosphate-buffered saline (PBS), membranes were incubated with one of the following primary antibodies: anti-PKC α (EMD Millipore, Billerica, MA), anti-PKC ϵ (Santa Cruz Biotechnology), anti-PKC ι (Abcam, Cambridge, MA), anti-vinculin (Sigma-Aldrich, St. Louis, MO), anti-PKC δ , anti-vimentin, anti-E-cadherin, anti-Snail, or anti-phospho-Smad2 (Cell Signaling Technology, Danvers, MA). We used either anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) as secondary antibodies. Bands were visualized by the enhanced chemiluminescence Western blotting detection system, and images were captured using a Fujifilm LAS-3000 system (Tokyo, Japan).

RNA Interference. RNA interference (RNAi) duplexes for silencing PKC α were purchased from Dharmacon (Lafayette, CO). The target sequences were as follows: PKC α RNAi 1, CCAUCCGCUCCACACUAAA; and PKC α RNAi 2, GAACAAGGAAUGACUU (Oliva et al., 2008). Control silencer RNAi was purchased from Ambion (Austin, TX). For transfection of RNAi duplexes (25 nM), we used Lipofectamine RNAi/MAX (Invitrogen, Carlsbad, CA).

Adenoviral Infections. Cells were infected with adenoviruses (AdVs) for PKC α , PKC δ , or LacZ (control) using different multiplicities of infection (MOIs), as previously described (Oliva et al., 2008). Adenoviral infections were carried out in RPMI 1640 medium supplemented with 2% fetal bovine serum. Four hours later, complete medium was added. Experiments were carried at different times after infection, as indicated.

Cell Viability Assay. Cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI), a colorimetric assay that contains MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and phenazine ethosulfate with enhanced chemical stability. Cells seeded into 96-well plates (1 $\times$ 10 4 cells/well) were treated with different concentrations of erlotinib for different times, as indicated. One hour after addition of the One Solution Reagent, absorbance was recorded at 490 nm using a 96-well plate reader.

Flow Cytometry. Subconfluent H1650 cells were detached using 0.02% EDTA in PBS, washed, pelleted, and resuspended in FACS buffer (PBS, pH 7.2, 0.2% bovine serum albumin). Then, 5 $\times$ 10 6 cells were costained with phycoerythrin-conjugated anti-human CD24 and allophycocyanin-conjugated anti-human CD44 antibodies (BD Biosciences, San Jose, CA). Labeling was performed for 1 hour at room temperature in the dark. Labeled cells were washed three times with the FACS buffer and sorted using a BD FACS Aria II cell sorter. Gates were set either at high or low expressions for CD24 and CD44, and subpopulations of cells were collected in FACS buffer for RNA extraction.

Statistical Analysis. All statistical analyses were done using GraphPad Prism software (version 5.03; GraphPad Software, San Diego, CA). Data were analyzed using a two-way analysis of variance. A *P* value <0.05 was considered statistically significant.

Results

Erlotinib-Resistant Cells Display Altered Expression of PKC Isozymes.

Changes in the expression levels of PKC isozymes have been associated with the progression of many types of cancers, including lung cancer, as well as with resistance to chemotherapeutic agents (Basu et al., 1996; Clark et al., 2003; Bae et al., 2007; Felber et al., 2007; Garg et al., 2014). To determine whether PKC isozymes are implicated in erlotinib resistance, we took advantage of a well characterized isogenic NSCLC cell model: the parental H1650 cell line and its erlotinib-resistant derivative H1650-M3 (Yao et al., 2010) (Fig. 1A). H1650 cells express cPKC α , nPKC δ , nPKC ϵ , and aPKC ζ . Western blot analysis revealed a remarkable upregulation of PKC α in erlotinib-resistant H1650-M3 cells. H1650-M3 cells also have reduced PKC δ levels relative to parental H1650 cells. A slight reduction in PKC ϵ levels was also observed in H1650-M3 cells (Fig. 1B). Densitometric analysis revealed the following levels relative to parental H1650 cells ($n = 3$): 10.90 ± 1.61 (PKC α), 0.40 ± 0.07 (PKC δ), 0.85 ± 0.06 (PKC ϵ), and 1.08 ± 0.13 (PKC ζ). Determination of mRNA levels for PKC α and PKC δ led to similar conclusions. Indeed, H1650-M3 cells have 25-fold higher PKC α mRNA levels than parental H1650 cells, whereas PKC δ mRNA levels are reduced by 5-fold in the erlotinib-resistant cell line (Fig. 1C).

PKC α Is Required But Not Sufficient to Induce Erlotinib Resistance. To assess a potential association between altered PKC α expression and erlotinib resistance, we used both pharmacological and RNAi approaches. Because PKC α has been implicated in drug resistance in some cancer types (Chen et al., 2010; Lee et al., 2012; Zhao et al., 2012) and

its levels are strikingly high in erlotinib-resistant cells, we speculated that this PKC could be involved in acquired resistance to erlotinib in NSCLC cells. Initial experiments showed that treatment of H1650-M3 cells with the pan-PKC inhibitor GF109203X increases their sensitivity to erlotinib ($10 \mu\text{M}$) (Fig. 2A). Gö6976, which preferentially inhibits cPKCs (Martiny-Baron et al., 1993), also enhanced the killing effect of erlotinib in H1650-M3 cells (Fig. 2B). PKC α is the most upregulated cPKC in this cell line; thus, it is likely that this PKC mediates erlotinib resistance.

To unambiguously establish a role for PKC α in erlotinib resistance, we used RNAi. Two different PKC α RNAi duplexes were transfected into H1650-M3 cells, which depleted PKC α by 91% (PKC α 1 RNAi) and 89% (PKC α 2 RNAi) relative to a nontarget control RNAi duplex, as determined by densitometry (Fig. 2C, left panel). A dose-response analysis for inhibition of cell viability by erlotinib revealed an IC_{50} of approximately $5 \mu\text{M}$ in nontarget control H1650 cells (which is similar to parental H1650 cells). On the other hand, IC_{50} in H1650-M3 cells was $>20 \mu\text{M}$, as also established in a previous study (Yao et al., 2010). Notably, PKC α depletion sensitizes H1650-M3 cells to erlotinib, as judged by the reduction in IC_{50} ($8.7 \pm 1.4 \mu\text{M}$ for PKC α 1 RNAi and $9.2 \pm 3.0 \mu\text{M}$ for PKC α 2 RNAi) (Fig. 2C, right panel).

To determine whether PKC α upregulation was sufficient to induce erlotinib resistance, PKC α was overexpressed in parental H1650 cells using an AdV. A LacZ AdV was used as control (Fig. 2D, left panel). We found that PKC α overexpression failed to alter the response of H1650 cells to the TKI ($\text{IC}_{50} = 4.7 \pm 1.3 \mu\text{M}$ for PKC α AdV and $5.5 \pm 2.0 \mu\text{M}$ for LacZ AdV) (Fig. 2D, right panel). Taken together, these data indicate that although PKC α is required for the resistance of NSCLC cells to erlotinib, overexpression of this kinase is not alone sufficient to induce erlotinib resistance.

PKC δ Alters the Sensitivity of H1650-M3 Cells to Erlotinib. Our results clearly ascribe a role for PKC α in determining the sensitivity of H1650 cells to erlotinib. The fact that H1650-M3 cells display PKC δ downregulation relative to parental H1650 cells prompted us to investigate whether changes in PKC δ levels could also dictate the sensitivity to the TKI. PKC δ was previously shown to mediate the cytotoxic effect of several anticancer drugs (Reyland et al., 1999; Blass et al., 2002). To address this issue, we first overexpressed PKC δ in H1650-M3 cells using a PKC δ AdV (Fig. 3A). As shown in Fig. 3B, overexpression of PKC δ in erlotinib-resistant cells caused a reduction in the IC_{50} for erlotinib. This effect was proportional to the expression levels of PKC δ achieved by infecting cells with different MOIs of the PKC δ AdV. Infection of H1650-M3 cells with an MOI equal to 1 plaque-forming unit/cell did not cause any significant PKC δ overexpression or sensitization to erlotinib ($\text{IC}_{50} = 24.2 \pm 0.6 \mu\text{M}$ for PKC δ AdV and $24.7 \pm 2.0 \mu\text{M}$ for control LacZ AdV). On the other hand, infection with PKC δ AdV at MOI = 10 plaque-forming units/cell caused significant sensitization ($\text{IC}_{50} = 8.7 \pm 1.9 \mu\text{M}$ for PKC δ AdV and $26.4 \pm 0.4 \mu\text{M}$ for LacZ AdV). At higher MOIs, the sensitivity of H1650-M3 cells was essentially similar to that observed in parental H1650 cells (MOI = 30: $\text{IC}_{50} = 6.3 \pm 0.5 \mu\text{M}$ for PKC δ AdV and $22.2 \pm 0.4 \mu\text{M}$ for LacZ AdV; MOI = 100: $\text{IC}_{50} = 4.5 \pm 0.4 \mu\text{M}$ for PKC δ AdV and $19.5 \pm 1.0 \mu\text{M}$ for LacZ AdV). Thus, PKC δ downregulation in H1650-M3 cells contributes to erlotinib resistance.

Fig. 1. Altered expression of PKC isozymes in erlotinib-resistant NSCLC cells. (A) Parental (H1650) and erlotinib-resistant (H1650-M3) cells were treated with erlotinib at indicated concentrations and cell viability was determined 24 hours later using an MTS assay. (B) Expression of PKC isozymes in parental (H1650) and erlotinib-resistant (H1650-M3) cells was analyzed by Western blotting. Similar results were observed in three individual experiments. (C) PKC α and PKC δ mRNA levels in H1650 and H1650-M3 cells were measured by qPCR. Human 18S rRNA was used as an endogenous control for normalization. Results (relative to H1650 cells) are expressed as the mean $\pm$ S.D. of triplicate samples. Similar results were observed in three additional experiments. ** $P < 0.01$; *** $P < 0.001$.

Fig. 2. PKC α protects H1650-M3 cells from erlotinib-induced cell death. (A) H1650-M3 cells were pretreated for 1 hour with either the pan-PKC inhibitor GF109203X (5 μ M) or vehicle. Cells were then treated with erlotinib (10 μ M), and cell viability was determined 24 hours later using an MTS assay. ** P < 0.01 versus vehicle. (B) H1650-M3 cells were pretreated for 1 hour with either the cPKC inhibitor G66976 (5 μ M) or vehicle. Cells were then treated with erlotinib (10 μ M), and cell viability was determined 24 hours later using an MTS assay. *** P < 0.001 versus vehicle. (C) H1650-M3 cells were transfected with either PKC α (PKC α 1 or PKC α 2) or nontarget control RNAi duplexes. After 48 hours, cells were treated with erlotinib for 24 hours at the indicated concentrations. The left panel shows PKC α expression by Western blot analysis. The right panel shows cell viability determined using an MTS assay. Parental H1650 cells were included for comparison. (D) Parental H1650 cells were infected with either PKC α AdV or LacZ AdV (MOI = 30 pfu/cell). Five days after infection, cells were treated with erlotinib at the indicated concentrations. The left panel shows PKC α expression by Western blot analysis. The right panel shows cell viability determined 24 hours later. H1650-M3 cells were included for comparison. Data are expressed as the mean $\pm$ S.D. of triplicate samples. Similar results were observed in two additional experiments. NTC, nontarget control.

Previous studies have shown that overexpression of one PKC isozyme could alter the expression of other PKC family members. For example, overexpression of PKC α alters the expression of PKC δ and PKC ϵ in various cellular models (Ways et al., 1995; Romanova et al., 1998; Tonetti et al., 2000). Because erlotinib-resistant H1650 cells display PKC α overexpression and PKC δ downregulation relative to the parental cell line, we asked whether there is a mutual regulation between these PKCs. To test our hypothesis, we either overexpressed PKC α or depleted PKC δ in parental H1650 cells. Interestingly, PKC α overexpression by adenoviral means reduced PKC δ expression, both at mRNA and protein levels. These effects were proportional to the PKC α overexpression levels achieved by using increased MOIs of the PKC α AdV (Fig. 4, A and B). Next, to assess whether downregulation of PKC δ alters PKC α expression levels, we silenced PKC δ expression from parental H1650 cells using RNAi. As shown in Fig. 4C, both control and PKC δ -depleted H1650 cells display similar PKC α levels. Furthermore, adenoviral overexpression of PKC δ in erlotinib-resistant H1650-M3 cells failed to induce changes in PKC α expression (Fig. 4D). These results argue for a unidirectional crosstalk whereby overexpression of PKC α in erlotinib-resistant H1650-M3 cells contributes to PKC δ downregulation; however, PKC δ was unable to influence PKC α expression.

PKC α Is Required for the Maintenance of Mesenchymal Phenotype of H1650-M3 Cells. Erlotinib-resistant H1650 cells exhibit mesenchymal properties, driven by the TGF- β pathway (Yao et al., 2010). The mesenchymal phenotype is a hallmark of cancer cells exhibiting an aggressive phenotype (Tam et al., 2013). A recent study in breast cancer showed that PKC α is upregulated in cells that had undergone EMT (Tam et al., 2013). Thus, we speculated that this kinase might contribute to the maintenance of the mesenchymal phenotype of erlotinib-resistant H1650 cells.

First, we investigated whether PKC α levels were elevated in a subpopulation of H1650 cells that display stem cell-like properties. Parental H1650 cells were sorted into CD44^{high}/CD24^{low} and CD44^{low}/CD24^{high} enriched populations, and PKC α mRNA levels were determined by qPCR. These experiments revealed PKC α upregulation in CD44^{high}/CD24^{low} cells (Fig. 5A). As shown in a previous study (Yao et al., 2010), H1650-M3 cells display elevated levels of genes associated with EMT, including vimentin, Snail, Twist, and Zeb2, as well as reduced levels of E-cadherin. To establish a potential link between PKC α upregulation and the mesenchymal phenotype of H1650-M3 cells, we examined the expression of EMT markers by qPCR after silencing PKC α . Notably, PKC α RNAi depletion caused a significant reduction in vimentin, Snail, Twist, and Zeb expression, suggesting that PKC α mediates the induction of these EMT

Fig. 3. PKC δ alters the sensitivity of H1650-M3 cells to erlotinib. (A) H1650-M3 cells were infected with either PKC δ AdV or LacZ AdV at the indicated MOIs. Expression of PKC δ was determined using Western blot analysis. Densitometric analysis is shown as the mean $\pm$ S.D. ($n = 3$). (B) A viability assay using MTS was carried out 48 hours after infection. Data are expressed as the mean $\pm$ S.D. of triplicate samples. Similar results were observed in two additional experiments. pfu, plaque-forming unit.

genes. Expression of the epithelial marker E-cadherin, however, remained unaffected (Fig. 5B). Changes were also validated at the protein level for those markers that could be readily detected by Western blot analysis (64 and 69% reduction for vimentin; 42 and 60% reduction for Snail, using PKC α 1 and PKC α 2 RNAi, respectively) (Fig. 5C). Despite the PKC α requirement for the expression of EMT markers in H1650-M3 cells, it became apparent that overexpression of this kinase in parental H1650 cells was not sufficient to induce these EMT genes, as determined by qPCR 72 hours after infection with increasing MOIs of the PKC α AdV (Fig. 5D). No changes were observed even 1 week after PKC α AdV infection (data not shown). Altogether, these results indicate that PKC α is required for the expression of genes involved in the maintenance of the mesenchymal phenotype of erlotinib-resistant cells; however, its overexpression is not sufficient to induce this phenotypical change.

Next, we set to explore whether PKC δ has a role in the expression of genes associated with EMT transition. Because PKC δ is downregulated in H1650-M3 cells, we adenovirally overexpressed PKC δ in these cells and assessed the expression of EMT markers by qPCR. Unlike PKC α silencing, ectopic overexpression of PKC δ in H1650-M3 cells did not change the expression of vimentin, Twist, or Zeb2, although a reduction in Snail levels could be observed. Likewise, PKC δ overexpression did not affect E-cadherin mRNA levels (Fig. 6A).

In addition, we also found that PKC δ RNAi depletion from parental H1650 cells failed to change the expression of Snail and E-cadherin (Fig. 6B). Therefore, the involvement of PKC δ is only confined to erlotinib resistance but not to EMT.

PKC α Upregulation in Erlotinib-Resistant Cells Is Mediated by TGF- β . TGF- β has been widely implicated in EMT in multiple cancer types (Massagué, 2012; Moustakas and Heldin, 2012). It was previously established that activation of the TGF- β signaling pathway mediates EMT and erlotinib resistance in H1650 cells (Yao et al., 2010). On the basis of this premise, we sought to establish whether a causal relationship exists between TGF- β signaling and PKC α expression. H1650-M3 cells were treated with the TGF- β receptor inhibitor LY2109761 (4-[5,6-dihydro-2-(2-pyridinyl)-4H-pyrrolo[1,2-b]pyrazol-3-yl]-7-[2-(4-morpholinyl)ethoxy]-quinoline), and its efficacy to inhibit TGF- β signaling was confirmed by its ability to reduce Smad2 phosphorylation (Fig. 7A). PKC inhibitors GF109203X and Gö6976 did not affect Smad2 phosphorylation, suggesting that PKC does not affect the activation of this pathway. Notably, the TGF- β receptor inhibitor caused a time-dependent reduction in PKC α mRNA level. This effect became noticeable at the protein level 48 and 72 hours after LY2109761 treatment (Fig. 7B). Furthermore, when parental H1650 cells were treated with TGF- β for different times, significant PKC α upregulation both at mRNA and protein levels could be observed. This

Fig. 4. PKC α modulates the expression of PKC δ in H1650 cells. (A) H1650 cells were infected with either PKC α AdV or LacZ AdV at the indicated MOIs. PKC α and PKC δ mRNA levels were determined by qPCR 72 hours after infection. Data are expressed as the mean $\pm$ S.D. of triplicate samples. Results are expressed as the fold change relative to LacZ AdV. (B) Expression of PKC α and PKC δ was determined by Western blot 72 hours after infection with either PKC α AdV or LacZ AdV. (C) Parental H1650 cells were transfected with either PKC δ (PKC δ 1 or PKC δ 2) or NTC RNAi duplexes. PKC α and PKC δ levels were analyzed 72 hours later by Western blotting. Similar results were observed in three independent experiments. Densitometric analysis is shown as the mean $\pm$ S.D. ($n = 3$). NTC, nontarget control.

effect was quite remarkable after long-term treatment with TGF- β (Fig. 7, C and D). Therefore, TGF- β signaling is implicated in the overexpression of PKC α observed in erlotinib-resistant cells.

Finally, we sought to establish an association between PKC α upregulation and TGF- β signaling in the induction of the mesenchymal phenotype. H1650 cells were infected with PKC α AdV (or LacZ AdV as a control) and then subjected to TGF- β treatment. mRNA was extracted 1 week after treatment and EMT markers were determined by qPCR. As shown in Fig. 7E, overexpression of PKC α potentiated TGF- β induction of vimentin, Snail, and Twist, thus establishing the relevance of the TGF- β /PKC α pathway in the induction of the mesenchymal phenotype.

Discussion

Tumor cells harboring activating mutations of EGFR are addicted to this oncogenic stimulus to maintain their proliferative and survival advantages. TKIs such as erlotinib are effective for treatment of advanced NSCLC tumors harboring EGFR-activating mutations. However, many patients treated with erlotinib develop resistance to the targeted molecular therapy (Tang et al., 2013; Steins et al., 2014). PKC isozymes have been recognized as key effectors of known oncogenes

implicated in drug resistance such as *c-MET*, *KRAS*, and *TGF- β* (Kermorgant et al., 2004; Sakaguchi et al., 2004; Symonds et al., 2011). Moreover, phorbol esters, which are known activators of PKCs, induce multidrug resistance (Fine et al., 1988; Kalalinia et al., 2012).

Here, we present evidence for the involvement of specific PKC isozymes in erlotinib resistance and EMT in NSCLC cells. Using an isogenic cell model, we found considerable changes in the expression of PKC isozymes that are causally associated with resistance to erlotinib. Erlotinib-resistant H1650-M3 cells exhibit elevated PKC α levels, whereas PKC δ expression in these cells is markedly downregulated. Although this is the first evidence for the involvement of these two PKC isozymes in resistance to this targeted molecular therapy, altered expression of PKC α and PKC δ has been detected in several cancer cell types. For example, elevation of PKC α expression or activity has been reported in pancreatic, colon, prostate, glioma, and gastric cancer cells resistant to chemotherapeutic drugs, including cisplatin, doxorubicin, and vincristine (Matsumoto et al., 1995; Wu et al., 2009; Chen et al., 2010; Zhao et al., 2012). Interestingly, comparable to what we observed in erlotinib-resistant cells, continuous exposure of MCF-7 breast cancer cells to tamoxifen rendered high levels of PKC α and downregulation of PKC δ (Li et al., 2012).

Fig. 5. PKC α is required for the expression of markers of the mesenchymal phenotype. (A) Parental H1650 cells were sorted into CD44^{high}/CD24^{low} and CD44^{low}/CD24^{high} subpopulations by flow cytometry. PKC α mRNA levels were determined by qPCR. Data are expressed as the mean $\pm$ S.D. of triplicate samples. (B) H1650-M3 cells were transfected with either PKC α (PKC α 1 or PKC α 2) or NTC RNAi duplexes. After 72 hours, RNA was extracted for qPCR analysis of selected genes associated with epithelial (E-cadherin) or mesenchymal (vimentin, Snail, Twist, and Zeb2) phenotypes. Results are shown as the fold change relative to parental H1650 cells. Data were expressed as the mean $\pm$ S.D. of triplicate samples. (C) Expression of epithelial and mesenchymal markers was determined by Western blot analysis. (D) H1650 cells were infected with either PKC α AdV or LacZ AdV at the indicated MOIs. After 7 days, expression of E-cadherin, vimentin, Snail, Twist, and Zeb2 were determined by qPCR. Similar results were observed in three independent experiments. NTC, nontarget control.

Studies have indicated the importance of PKC α overexpression in protecting cancer cells against drug-induced cell death. For example, PKC α overexpression in colon cancer cells attenuates doxorubicin-induced apoptosis by elevating phosphorylation of Bcl-2, Bad, and decreasing PARP cleavage. More importantly, in several cancer models, PKC α overexpression has been associated with increased drug resistance by elevating expression and phosphorylation of the drug efflux pump P-glycoprotein encoded by the multidrug resistant gene *MDR1* (Lee et al., 2012). The functional importance of PKC α overexpression has been further demonstrated by using

pharmacological inhibitors and RNAi. For example, inhibition of PKC α using Gö6976 restores the sensitivity of pancreatic cancer cells to chemotherapeutic drugs (Chen et al., 2010), and silencing PKC α by RNAi reverses drug resistance in ovarian cancer cells (Zhao et al., 2012). In our study, we found that RNAi depletion or inhibition of PKC α using Gö6976 sensitizes erlotinib-resistant NSCLC cells to the TKI.

As previously characterized, H1650-M3 cells have elevated expression of genes associated with EMT and display morphological changes that are reminiscent of the mesenchymal

Fig. 6. Genes involved in the mesenchymal phenotype are not regulated by PKC δ . (A) H1650-M3 cells were infected with either PKC δ AdV or LacZ AdV (MOI = 100 pfu/cell). After 96 hours, mRNA levels for various mesenchymal (vimentin, Snail, Twist, and Zeb2) or epithelial (E-cadherin) associated genes were measured by qPCR. Results are shown as the fold change relative to control (LacZ AdV-infected) H1650-M3 cells. Data were expressed as the mean $\pm$ S.D. of triplicate samples. (B) Parental H1650 cells were transfected with either PKC δ (PKC δ 1 or PKC δ 2) or NTC RNAi duplexes. Expression of PKC δ , E-cadherin, and Snail was analyzed by Western blotting 72 hours later. Similar results were observed in three independent experiments. NTC, nontarget control; pfu, plaque-forming unit.

Fig. 7. TGF- β signaling controls PKC α expression in erlotinib-resistant cells. (A) H1650-M3 cells were pretreated for 1 hour with either the pan-PKC inhibitor GF109203X (5 μ M), the cPKC inhibitor G66976 (5 μ M), the TGF- β receptor inhibitor LY2109761 (5 μ M), or vehicle. Cells were then treated with TGF- β (20 ng/ml, 30 minutes) and phospho-Smad2 levels were determined by Western blot analysis. (B) H1650-M3 cells were treated with the TGF- β receptor inhibitor LY2109761 (5 μ M) for the indicated times. PKC α mRNA and protein levels were determined by qPCR and Western blot analysis, respectively. Densitometric analysis is shown as the mean $\pm$ S.D. (n = 3). (C) PKC α mRNA levels in H1650 cells were measured 6 hours or 2 weeks after TGF- β treatment. (D) H1650 cells were treated with TGF- β (5 ng/ml) for 24 hours, 48 hours, 1 week, or 2 weeks. PKC α levels were determined by Western blot analysis. Densitometric analysis is shown as the mean $\pm$ S.D. (n = 3). (E) H1650 cells were infected with either PKC α AdV or LacZ AdV (MOI = 30 pfu/cell). Twenty-four hours after infection, cells were treated with TGF- β (5 ng/ml) for 1 week. mRNA levels for PKC α , Snail, vimentin, and Twist were measured using qPCR. In all cases, data were expressed as the mean $\pm$ S.D. of triplicate samples and experiments were reproduced at least three times. pfu, plaque-forming unit.

phenotype. Interestingly, parental erlotinib-naïve cells possess a small subpopulation of cells that are mesenchymal, erlotinib resistant, and similar to H1650-M3 cells (Yao et al., 2010), indicating that H1650-M3 cells were potentially generated through a selection process that favors the survival of cells that use alternate mechanisms to overcome drug-induced death. A recent study by the Weinberg laboratory established that PKC α preferentially supports the maintenance of the mesenchymal cell state through the regulation of the Fos-related antigen 1 transcription factor. In addition, elevated PKC α expression was found in a subpopulation of normal mammary epithelial cells enriched in the mesenchymal surface marker CD44 (Tam et al., 2013). Similarly, our results indicate a correlation between enrichment of the mesenchymal phenotype and PKC α expression in NSCLC cells. Inhibition of PKC α in H1650-M3 cells also led to a reduction in the expression of genes associated with the mesenchymal phenotype. Interestingly, although exposure to erlotinib resulted in a differential expression of EMT markers, including upregulation of vimentin, Snail, Twist, and Zeb2, as well as downregulation of E-cadherin, the effect of inhibiting PKC α was limited to the genes associated with the mesenchymal phenotype, thus underscoring its role in the maintenance of this phenotype.

In our study, we also identified a functional link between TGF- β and PKC α . TGF- β signaling was shown to be sufficient and required for the induction of erlotinib resistance and EMT in H1650-M3 cells (Yao et al., 2010). We found that inhibition of TGF- β signaling reduced the expression of PKC α in H1650-M3 cells. On the other hand, TGF- β increased the expression of PKC α in parental H1650 cells, indicating that in the process of acquiring an aggressive phenotype, TGF- β upregulates the expression of PKC α . TGF- β is known to control gene expression by activating the Smad transcription factors (Massagué, 2012). The promoter region of PKC α does not display any obvious Smad binding site (data not shown), arguing for the involvement of alternative or indirect mechanisms. It is worth noting that gene profiling analysis in A549 lung adenocarcinoma cells identified PKC α as a TGF- β target gene (Ranganathan et al., 2007).

In summary, our results provide evidence for a role of PKCs in acquired drug resistance to erlotinib and EMT. Elevation of PKC α expression as well as PKC α -dependent downregulation of PKC δ are required for erlotinib resistance, whereas mesenchymal genes are regulated only by PKC α . Our results argue for a potential therapeutic use of PKC α inhibitors to overcome drug resistance and EMT in lung cancer.

Authorship Contributions

Participated in research design: Abera, Kazanietz.

Conducted experiments: Abera.

Performed data analysis: Abera, Kazanietz.

Wrote or contributed to the writing of the manuscript: Abera, Kazanietz.

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