Signaling and Regulation

Activation by Tyrosine Phosphorylation as a Prerequisite for Protein Kinase Cζ to Mediate Epidermal Growth Factor Receptor Signaling to ERK

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Abstract

The atypical protein kinase Cζ (PKCζ) was recently shown to mediate epidermal growth factor (EGF)-induced activation of extracellular signal–regulated kinase (ERK) in head and neck squamous carcinoma (HNSCC) cells. Here, it is shown that EGF may induce tyrosine phosphorylation of PKCζ in several HNSCC cells, breast carcinoma cells, as well as mouse embryonic fibroblasts. In COS-7 cells overexpressing EGF receptor (EGFR) and PKCζ as a tumor cell model, we show that PKCζ tyrosine phosphorylation by EGF is induced by catalytic activation. Using a loss-of-function mutant of PKCζ, we can show that the tyrosine residue 417 in PKCζ plays an important role in both PKCζ activation and the ability of PKCζ to mediate activation of ERK. The importance of PKCζ in EGF-induced ERK activation can also be shown in several HNSCC and breast carcinoma cell lines as well as in PKCζ-deficient mouse embryonic fibroblasts. In addition, we present several lines of evidence suggesting the physical association of PKCζ with EGFR and the importance of the EGFR tyrosine kinase c-Src and the Src-specific phosphorylation site pY845-EGFR in the tyrosine phosphorylation as well as catalytic activation of PKCζ. This study characterizes PKCζ as a novel mitogenic downstream mediator of EGF and indicates PKCζ as a therapeutic target in some carcinomas. Mol Cancer Res; 8(5); 783–97. ©2010 AACR.

Introduction

Alterations of ErbB family receptors and/or their signaling pathways are involved in the pathogenesis and progression of various human carcinoma types such as breast, lung, colon, and head and neck cancers (1-3). In head and neck squamous cell carcinoma (HNSCC), for example, which is one of the most common cancers worldwide, the epidermal growth factor receptor (EGFR, ErbB1) as well as its ligand, transforming growth factor α, are upregulated, leading to an aggressive behavior of HNSCC. Therefore, overexpressed EGFR represents a marker of poor prognosis and a therapeutic target in the treatment of HNSCC (4).

Focusing on HNSCC cells, there is accumulating evidence that several pathways downstream of the EGFR may play a critical role in cancer development. Thus, activation of extracellular signal–regulated protein kinase (ERK) seems to be required for proliferation, but not for invasion, of HNSCC cells (5). In contrast, invasion seems to be dependent on EGFR-induced activation of phospholipase Cγ1 (6). EGFR signaling through the phosphoinositide 3-kinase/Akt pathway may promote survival, proliferation, invasion, as well as angiogenesis and was used as an attractive therapeutic target in HNSCC (7, 8). In addition, signal transducer and activator of transcription (STAT) proteins, including STAT3 and STAT5b, have been described to contribute to the EGFR-mediated oncogenic process in HNSCC (9, 10). Recently, a novel mitogenic pathway including protein kinase Cζ (PKCζ) has been reported that mediates EGF-induced activation of mitogen-activated protein kinase (MAPK) and MAPK-dependent proliferation in several HNSCC cell lines (11). Whereas the Ras-independent activation of ERK by PKCζ as well as by other PKC isoforms has been repeatedly described (12, 13), the molecular mechanism of how PKCζ may be activated downstream of the EGFR is completely unknown.

The PKC isozymes constitute a family of more than 10 serine/threonine kinases that are involved in a variety of signaling pathways, including those of G-protein–coupled receptors and receptor tyrosine kinases. They mediate a broad diversity of physiologic processes and play a critical role in cell proliferation, differentiation, tumorigenesis, and apoptosis in many cell types. PKCs have been classified into three distinct subfamilies: the Ca2+--, phospholipid-, and diacylglycerol-dependent (conventional) cPKCs...
(α, β, γ); the Ca²⁺-independent and diacylglycerol-dependent (novel) δ PKCs (δ, e, η, θ); and the (atypical) αPKCs (α, ζ), which are also Ca²⁺ independent but do not require diacylglycerol for activation (14). These atypical PKCs reveal an atypical NH2-terminal structure and a relatively low sequence homology with the other PKC isoforms. Thus, PKCζ consists of four functional domains and motifs, including a PB1 domain in the NH2 terminus, a pseudosubstrate (PS) sequence, a cysteine-rich C1 domain of a single zinc finger motif, and a COOH-terminal kinase domain including an ATP-binding region, an activation loop, and a turn motif. PKCζ can be directly or indirectly activated by different signaling mechanisms (15). Phosphorylation of Thr⁴¹₀ in the activation loop and the subsequent autophosphorylation at Thr⁵⁶⁰ in the turn motif are essential for PKCζ activation (16). The initial phosphorylation at Thr⁴¹₀ is mediated by phosphoinositide-dependent kinase 1 (PDK1) through a pathway including the activation of phosphatidylinositol 3,4,5-trisphosphate, which activates PDK1. Alternatively, phosphatidylinositol 3,4,5-trisphosphate can also directly interact with PKCζ and release the PS-dependent autoinhibition. Obviously, both effects of phosphatidylinositol 3,4,5-trisphosphate are necessary for the full activation of PKCζ (15, 17). Recently, phospholipase D2 has been identified to interact with PKCζ through protein-protein interaction leading to direct activation of PKCζ and an increase in activation loop phosphorylation (18). Alternatively, tyrosine phosphorylation might be another, but less well-characterized, mode of PKCζ activation and was mainly reported for PKCh (19) and PKCd (20). Thus, the in vitro tyrosine phosphorylation of recombinant PKCζ has been shown. Based on phosphorylation probability analysis as well as homology structural modeling, the Tyr⁴⁵² in PKCζ in the catalytic loop was predicted as the site to be phosphorylated (21).

Like EGFR, PKCζ may be also upregulated in several cell types, such as chondrocytes (22) or fibroblasts (23), and may occur in high expression levels in various cancer cells [e.g., different HNSCC cells (11); many breast carcinoma cells such as MCF-7, T47D, or MDA-MB 468 (24, 25); and small-cell lung cancer and non–small-cell lung cancer cells (26)].

To mimic these conditions existing endogenously in carcinoma cells, we used a PKCζ/EGFR overexpression strategy as an approach to investigate a putative role of PKCζ downstream of EGFR. In this model and also in several HNSCC and breast carcinoma cells, we can show that the activated EGFR mediates tyrosine phosphorylation of PKCζ. Using mouse embryonic fibroblast (Mef) cells deficient in PKCζ and a loss-of-function mutant, PKCζ-Y417F, we can show that tyrosine phosphorylation and thereby catalytic activation of PKCζ contributes to the EGF-induced activation of MAPK. Furthermore, we present several lines of evidence suggesting a critical role of the EGFR tyrosine kinase c-Src and the Src-specific EGFR phosphorylation site pY845-EGFR for tyrosine phosphorylation of PKCζ. Thus, our results define PKCζ as a novel downstream target of EGFR and provide some mechanistic explanation on the importance of PKCζ in MAPK activation by EGF.

Materials and Methods

Materials. COS-7 cells and the carcinoma cell lines T47D, MDA-MB 468, and FaDu were obtained from the American Type Culture Collection. The carcinoma cell lines MCF-7, MDA-MB 453, and A431 were from the German Collection for Microorganisms and Cell Cultures (DSMZ). The HNSCC cell line PE/CA-PJ-15 (European Collection of Cell Cultures; ECACC) was kindly provided by Dr. Alexander Berndt (Institute of Pathology, University of Jena, Jena, Germany). Mef cells deficient in PKCζ (PKCζ−/− Mef cells) and Mef wild-type (WT) cells were a generous gift of Dr. Michael Leitges (Biotechnology Centre of Oslo, Oslo, Norway). DMEM, FCS, Lipofectamine 2000, and streptavidin-agarose beads were purchased from Invitrogen. Enhanced chemiluminescence detection reagent, protein A-Sepharose, and Hybond polyvinylidene difluoride membrane were purchased from Amersham Biosciences. Antibodies against PKCζ, PKCδ, ERK1 and ERK2, actin, c-Src, hemagglutinin (HA), phospho-tyrosine PY99, and phospho-Akt (Ser473); horseradish peroxidase–conjugated secondary antibodies; and rabbit and mouse IgG antibodies were from Santa Cruz Biotechnologies, Inc. Phospho-tyrosine 4G10 antibody was from Upstate. EGF antibody (clone 13G8) was purchased from Nano-Tools Antikörpertechnik. The monoclonal EGF antibody mAb 425 was a generous gift of Dr. A. Sutter (Merck, Darmstadt, Germany). The inhibitors AG1478, PP-2, and Myr-PS were from Calbiochem. [γ-32P]ATP was obtained from Perkin-Elmer Life Sciences. Myelin basic protein (MBP), EGF, and all other standard chemicals were from Sigma. Fmoc-amino acid derivatives, coupling reagents, and polymer support were purchased from Novabiochem and Orpegen.

Plasmids. Human PKCζ and PKCδ have been described previously (27). PKCζ-T410A-EYFP-N1 cells were kindly provided by Prof. K. Pfitzenmaier (University of Stuttgart, Stuttgart, Germany). EGFR-WT, EGFR-Y1173F, EGFR-Y992F, and EGFR-Y1086F were generously provided by Prof. F. Boehmer (University of Jena, Jena, Germany). EGFR-Y845F in pcDNA3.0 was a gift of Prof. S.J. Parsons (University of Virginia, Charlottesville, VA). EGFR-ΔCR1 (ΔA24-259) was kindly provided by Prof. A.W. Burgess (Ludwig Institute for Cancer Research, Melbourne, Australia). The c-Src dominant-negative mutant (SrcRF-K295R/Y527F) was a generous gift from Prof. Joan Brugge (Harvard Medical School, Boston, MA).

Cell culture, transfection, and cell lysis. Cells were maintained in DMEM supplemented with 10% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B. Subconfluent COS-7 cells were transfected by the DEAE-dextran technique or Lipofectamine 2000 as indicated using standard protocols. Two days after transfection, COS-7 cells were
FIGURE 1. Characterization of EGF-induced tyrosine phosphorylation of PKCζ. A, EGF-induced tyrosine phosphorylation of PKCζ in various carcinoma cell lines. Serum-starved cells were stimulated with 10 nmol/L EGF for 5 minutes. PKCζ was immunoprecipitated, analyzed by Western blotting with anti-phosphotyrosine antibody (4G10), and reprobed with anti-PKCζ antibody. For each lane, 100 μg of lysate protein from each cell line were loaded. Representative results of two to four separate experiments. Carcinoma cell lines: FaDu, human pharynx squamous cell carcinoma; PE/CA-PJ-15, human oral squamous cell carcinoma; MCF-7, human breast adenocarcinoma; T47D, human breast carcinoma; MDA-MB453, human breast carcinoma; MDA-MB468, human breast adenocarcinoma; A431, human epidermoid carcinoma. B, COS-7 cells were transiently transfected with either vector or PKCζ or cotransfected with PKCζ and EGFR. After serum starvation overnight, cells were stimulated with 10 nmol/L EGF for 5 minutes. PKCζ was immunoprecipitated (IP) and analyzed by Western blotting (WB) with anti-phosphotyrosine antibody and reprobed with anti-PKCζ antibody. In aliquots of the cell lysates, the expression level of EGFR was determined by Western blotting with anti-EGFR antibody (13G8). Representative results of three independent experiments. C and D, time course curve (C) and dose-response curve (D) of EGF-stimulated tyrosine phosphorylation of PKCζ in COS-7 cells. Only PKCζ in C and PKCζ and EGFR in D are overexpressed. Shown are representative data. The curves are the mean of three (C) or four (D) separate experiments and expressed as percentage of PKCζ tyrosine phosphorylation in response to EGF compared with nonstimulated cells; bars, SE. The data have been normalized to the levels of PKCζ immunoprecipitates.
serum starved overnight, pretreated with inhibitors or with a vehicle, and then stimulated with epidermal growth factor (EGF) as indicated, washed in cold PBS, and lysed at 4°C in a buffer containing 20 mmol/L HEPES (pH 7.5), 10 mmol/L EGTA, 40 mmol/L β-glycerophosphate, 1% Triton X-100, 2.5 mmol/L MgCl₂, 1 mmol/L DTT, 2 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 20 μg/mL aprotinin, and 20 μg/mL leupeptin. Lysates were centrifuged at 14,000 × g for 5 minutes at 4°C.

Immunoprecipitation and Western blotting. Lysates were incubated with the indicated antibodies for 3 hours at 4°C, and immune complexes were recovered with protein A-Sepharose for an additional hour at 4°C. Beads were washed three times with PBS supplemented with 1% Triton X-100 and 2 mmol/L sodium orthovanadate and boiled in Laemmli buffer, and the supernatants were resolved by SDS-PAGE on 8% to 10% gels and then transferred onto polyvinylidene difluoride membranes. Proteins were detected with the respective antibodies, as indicated, followed by enhanced chemiluminescence detection. Expression levels of the cDNA constructs were verified by Western blotting with the appropriate antibodies. The radioactive signals (Fig. 4A) were quantified with a phosphoimager. Densitometric analysis of immunoblots was done with NIH Image 1.61 software. The pERK1/2 bands were quantified separately and then combined.

Peptide synthesis and purification. The peptide Btn-Ado-GAEEKEYHAEGGK-NH₂ (EGF-Y845) was synthesized in tyrosine-phosphorylated and nonphosphorylated forms by solid-phase peptide synthesis according to the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy as recently described (28). The molecular weights of the peptides were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Laser Tec Research mass spectrometer (Perseptive Biosystems).

In vitro pull-down assays. COS-7 cells overexpressing either PKCζ or PKCa were lysed. Equal amounts of protein from each sample were incubated overnight with 10 μg of the respective biotinylated peptides at 4°C. Then, 20 μL of streptavidin-agarose beads were added, followed by incubation at 4°C for 1 hour. Protein complexes were pulled down by centrifugation, washed twice with lysis buffer, and boiled...
in SDS sample buffer for 5 minutes. Proteins were analyzed by Western blotting using anti-PKCζ or anti-PKCα antibodies as indicated. The protein content was measured using the Roti-Nanoquant kit according to the manufacturer’s protocol.

**Site-directed mutagenesis.** PKCζ-Y417F was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the WT human PKCζ as a template. A pair of primers (35-mers) fully complementary to each other was designed to contain the mutation -Y417F (sense strand, 5'-ctgcggaaccccgaatttcatcgcccccgaaatcc-3') and Y417Fr (antisense strand, 5'-ggatttcgggggcgatgaaattcggggttcccgag-3'), and site-directed mutagenesis was carried out according to the manufacturer’s protocol.

**MAPK assay.** COS-7 cells were cotransfected with HA-MAPK (ERK2) and the constructs as indicated using the DEAE-dextran method. The MAPK assay was done as described previously (27). Phosphorylated MBP was visualized by autoradiography. The upper parts of the gels were transferred onto polyvinylidene difluoride membranes and analyzed with anti-HA antibody for the amount of immunoprecipitated HA-MAPK.

**PKCζ immune complex kinase activity assay.** The MBP kinase assay was done according to Cenni et al. (29). Kinase reactions were carried out in 50 μL of kinase buffer supplemented with 1 μCi of [γ-32P]ATP, 20 μmol/L unlabeled ATP, and 40 μg of MBP for 20 minutes at 37°C. Reactions were stopped by addition of Laemmli

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**FIGURE 2. Continued.** D, COS-7 cells cotransfected with EGFR and PKCζ as indicated were serum starved and stimulated with 10 nmol/L EGF for 5 minutes. PKCζ immunoprecipitates were assayed in the presence of [γ-32P]ATP for PKC activity using MBP as substrate. Top, an autoradiogram of MBP phosphorylation, which is representative of six independent experiments. Membranes were probed with anti-PKCζ antibody. Using aliquots of the lysates, the equal expression of EGFR was determined by Western blotting. E, quantified data from D, normalized to the PKCζ immunoprecipitates. The basal activity due to overexpression of PKCζ (100%) is significantly enhanced by both coexpression of EGFR and in response to EGF stimulation. Columns, means; bars, SD. *, significantly higher compared with basal activity; **, significantly higher compared with the basal activity without coexpressed EGFR; #, significantly higher than the EGF effect without coexpressed EGFR (P < 0.05). Data are from six independent experiments. F, COS-7 cells transfected with WT PKCζ alone were serum starved and pretreated with 10 nmol/L AG1478 for 30 minutes, and the endogenous EGFR was stimulated with 10 nmol/L EGF for 5 minutes. The immunoprecipitates of PKCζ were used for the immune complex kinase assay with MBP as substrate. The second row shows the inhibition of EGFR autophosphorylation by AG1478. The third row shows the consistent immunoprecipitation of PKCζ. Representative results of four independent experiments. G, quantification of the data in F normalized to the PKCζ immunoprecipitates. Columns, means; bars, SD. *, significantly higher compared with basal; +, significantly lower compared with EGF (P < 0.05).
The mutant PKCζ-Y417F reveals reduced tyrosine phosphorylation and is insensitive toward EGF-induced catalytic activation.

A, schematic depiction of the domain organization of PKCζ. In the kinase domain, the threonine residues involved in the activation steps by PDK1 and autophosphorylation and the position of site-directed mutagenesis are marked. B, COS-7 cells were cotransfected with EGFR and, selectively, WT PKCζ, the catalytically inactive mutant PKCζ-T410A (as negative control), or the novel mutant PKCζ-Y417F. The cells were serum starved and stimulated with 10 nmol/L EGF for 5 minutes. The PKCζ immunoprecipitates were assayed in the presence of [γ-32P]ATP for PKC kinase activity using MBP as substrate. Top, an autoradiogram of MBP phosphorylation, which is representative of six independent experiments. Membranes were probed with anti-PKCζ antibody revealing the quantity of immunoprecipitation. The equal expression of EGFR was determined by Western blotting using aliquots of the lysates. C, quantified data from B. The activity of WT PKCζ (100%) but not that of the mutants is significantly enhanced in response to EGF stimulation compared with the basal activity. Columns, means; bars, SD. *, significantly higher compared with the basal activity of the WT PKCζ.

D, COS-7 cells were cotransfected with EGFR and the PKCζ constructs as indicated. Serum-starved cells were treated with 10 nmol/L EGF for 5 minutes. The immunoprecipitates were analyzed with anti-phosphotyrosine antibody (4G10) and reprobed with anti-PKCζ antibody. Representative of six experiments. E, quantitative analysis of PKCζ tyrosine phosphorylation (data from D). Both basal and EGF-induced tyrosine phosphorylation of the mutants are compared with those of the WT PKCζ (basal value, 100%). Columns, means; bars, SD. *, significantly higher compared with the basal value; †, significantly lower compared with the basal value; ‡, significantly lower compared with the WT (P < 0.05). The data from C and E were normalized to the PKCζ rebots.
buffer, boiled for 5 minutes, separated on 13% SDS-polyacrylamide gels, and analyzed by autoradiography.

**[3H]Thymidine incorporation assay.** COS-7 cells were transfected in 24-well plates using Lipofectamine 2000 as indicated. Forty-eight hours after transfection, cells were serum starved for 24 hours. The cells were stimulated with 10 nmol/L EGF for 20 hours, followed by incubation with 1 μCi of [3H]thymidine per well for an additional 4 hours. Cells were washed with ice-cold PBS, incubated with 10% TCA trichloroacetic acid supplemented with 1% pyrophosphate (20 mmol/L) for 30 minutes on ice, and then subsequently washed twice with 1% TCA, 1% pyrophosphate, twice with 96% ethanol, followed by solubilization with 1 N NaOH for 10 minutes on ice with gentle shaking and neutralization with 2 N HCl. The incorporation of [3H]thymidine into newly synthesized DNA was measured in Lumasafe Plus on a Beckman scintillation counter.

**Results**

**EGF may induce tyrosine phosphorylation of PKCζ.** To investigate the molecular mechanism by which PKCζ might be able to mediate oncogenic EGF signaling, we considered recently published data suggesting a critical role of PKCζ in EGF-induced activation of MAPK in HNSCC (11) or chemotaxis in breast carcinoma (25) cells. We used these carcinoma cell types to investigate PKCζ tyrosine phosphorylation, and after treating the cells with 10 nmol/L EGF for 5 minutes, we found significant tyrosine phosphorylation of PKCζ in two HNSCC cell lines (FaDu and PE/Ca-PJ-15), three breast carcinoma cell lines (MCF-7, T47D, and MDA-MB-468), and, for comparison, A431 cells. It should be noted that the breast carcinoma cell line MDA-MB 453, which lacks EGFR, displays extremely high basal tyrosine phosphorylation of PKCζ (Fig. 1A). Next, we studied PKCζ tyrosine phosphorylation in COS-7 cells used as a transfection model. In these cells, due to low levels of endogenous PKCζ tyrosine phosphorylation by EGF was not detectable. In contrast, when we mimicked the conditions occurring in the majority of carcinoma cells by overexpressing PKCζ and EGFR, significant PKCζ tyrosine phosphorylation was detectable. When PKCζ and EGFR were cotransfected, we obtained an approximately 3.5-fold increase in PKCζ tyrosine phosphorylation by EGF compared with the basal level (Fig. 1B). The effect of EGF was very rapid, reaching a maximum after 1 minute, and PKCζ tyrosine phosphorylation was stable up to 20 minutes (Fig. 1C). Furthermore, the effect of EGF was concentration dependent and revealed an EC_{50} value of ~0.4 nmol/L (Fig. 1D).

**EGFR-mediated tyrosine phosphorylation of PKCζ leads to catalytic activation of PKCζ.** The EGF-induced tyrosine phosphorylation of PKCζ was completely prevented by the EGFR tyrosine kinase–specific inhibitor AG1478 (Fig. 2A). Furthermore, when the non-dimerizing ΔCR1 loop EGFR mutant (31) was overexpressed, PKCζ was not tyrosine phosphorylated by EGF (Fig. 2B). Coinmunoprecipitation studies were done to address the question whether tyrosine phosphorylation of PKCζ requires the direct interaction with the EGFR. The basal as well as EGF-induced association between PKCζ and EGFR is shown in the COS-7 cell transfection model and, for example, in FaDu and MDA-MB468 cells under endogenous conditions (Fig. 2C). Our findings suggest the critical role of EGFR in PKCζ tyrosine phosphorylation. Whereas under endogenous conditions there was no detectable activation of PKCζ, overexpression of PKCζ was sufficient for MBP phosphorylation. This low basal catalytic activity was increased by both overexpression of EGFR and stimulation with EGF (Fig. 2D and E). Again, the EGF-induced catalytic activation of PKCζ was prevented by AG1478, suggesting the critical involvement of EGFR tyrosine kinase in tyrosine phosphorylation as well as activation of PKCζ by EGF (Fig. 2F and G).

**Phosphorylation of the tyrosine residue 417 is involved in the EGFR-mediated catalytic activation of PKCζ.** Considering the hitherto existing data (21), we next generated a mutant, PKCζ-Y417F, where the tyrosine adjacent to the PDK1-phosphorylated Thr410 in the activation loop was replaced by phenylalanine (Fig. 3A). Tyr417 in PKCζ corresponds to Tyr512 in PKCδ wherein its replacement by phenylalanine abolished catalytic activity (32).

Stimulation with EGF leads to the catalytic activation of WT PKCζ (Fig. 3B and C; see also Fig. 2). As a negative control, we used the catalytically inactive mutant PKCζ-T410A, which was available as an EYFP-tagged construct. The tag had no importance for the present investigation. Surprisingly, the novel mutant PKCζ-Y417F, possessing the intact PDK1-phosphorylation site T410, was also not significantly activated by EGF (Fig. 3B and C). Interestingly, both mutants display different patterns of tyrosine phosphorylation. Compared with WT PKCζ, the basal (due to EGFR overexpression) tyrosine phosphorylation of the catalytically competent Y417F mutant is slightly lowered, but that of the catalytically incompetent mutant T410A is considerably decreased. In contrast, both mutants reveal a similar extent of reduction in tyrosine phosphorylation in response to EGF (Fig. 3D and E). This apparent discrepancy becomes more understandable when the EGF-induced tyrosine phosphorylation levels are compared with the respective basal levels of each individual PKCζ. In Fig. 3D it is shown that due to the lowered basal level, the T410A mutant is much more tyrosine phosphorylated by EGF than the Y417F mutant. The reasons for these discrepancies are not yet understood but conformational restrictions may be assumed. More importantly, compared with the WT, the novel mutant Y417F shows a tyrosine phosphorylation in response to EGF, which is reduced by approximately 30% to 40%.

**Phosphorylation of Y417 is also involved in PKCζ-induced activation of ERK.** In COS-7 cells overexpressing EGFR, EGF-induced activation of MAPK (ERK1/2) was significantly elevated when PKCζ was additionally transfected. This effect was not observed when the
WT was replaced by the catalytically inactive mutants PKC\(\zeta\)-T410A or PKC\(\zeta\)-Y417F (Fig. 4A and B). Additionally, we estimated the influence of PKC\(\zeta\) on DNA synthesis. Stimulation of vector-transfected COS-7 cells with 10 nmol/L EGF leads to an approximate doubling in DNA synthesis (Fig. 4C). Significant increase in \(^{3}\text{H}\)thymidine incorporation can also be observed due to overexpression of either EGFR or WT PKC\(\zeta\). Under these conditions, no further increase in DNA synthesis by additional EGF stimulation can be measured, suggesting that the experimental system is working to capacity. Coexpression of EGFR and WT PKC\(\zeta\) results in an additive effect on \(^{3}\text{H}\)thymidine incorporation. In contrast, coexpression of the PKC\(\zeta\)-Y417F mutant reduced DNA synthesis to the level of cells simply overexpressing EGFR. These findings suggest that on the EGFR module, PKC\(\zeta\) may be catalytically activated by tyrosine phosphorylation, subsequently mediating, at least in part, some mitogenic effects of EGF such as activation of ERK and cell proliferation. To determine whether PKC\(\zeta\) contributes to EGF-induced activation of ERK under endogenous conditions, various carcinoma cell lines were stimulated with EGF in the presence or absence of the specific PKC\(\zeta\) inhibitor Myr-PS. In three of the cell lines (FaDu, MDA-MB 468, and MCF-7) also revealing tyrosine phosphorylation of PKC\(\zeta\) (Fig. 1A), inhibition of PKC\(\zeta\) led to a significant decrease in EGF-induced ERK activation (Fig. 4D). The ERK activation of the other carcinoma cell lines shown in Fig. 1A, such as PE/Ca-PJ-15, MDA-MB 553, or A431, did not negatively respond toward Myr-PS (not shown). Alternatively to the COS-7 cell model, MCF-7 cells were transiently transfected with either WT PKC\(\zeta\) or the catalytically inactive mutant PKC\(\zeta\)-T410A or PKC\(\zeta\)-Y417F, and the EGF-induced activation of ERK was determined using pERK antibody. In Fig. 4E and F, it is shown that overexpression of PKC\(\zeta\)-WT results in additive ERK activation in response
to EGF. In contrast, this additional effect did not occur when the catalytically inactive mutants were overexpressed.

**ERK activation by EGF is reduced in PKCζ−/− Mef cells.** Next, we stimulated WT Mef cells expressing PKCζ with increasing concentrations of EGF. We found a significant and dose-dependent increase in tyrosine phosphorylation of PKCζ, revealing an EC50 value of 0.06 nmol/L (Fig. 5A and B). To determine whether the lack of PKCζ impairs ERK activation, both WT and PKCζ-deficient (PKCζ−/−) Mef cells were stimulated with EGF and the activation of ERK1/2 was measured. As shown in Fig. 5C and D, in Mef−/− cells lacking PKCζ, the EGF-induced activation of ERK was significantly diminished compared with the WT Mef cells expressing PKCζ. It should be noted that the basal activity of ERK is also slightly higher in WT cells than in PKCζ−/− cells. Both the WT and the PKCζ−/− Mef cells have been previously characterized in detail (33).

**Role of the Src-specific phosphorylation site EGFR-Y845 in EGFR-PKCζ association, EGF-induced tyrosine phosphorylation, as well as catalytic activation of PKCζ.** To further elucidate the mechanism of how the EGF may mediate tyrosine phosphorylation of PKCζ, additional coimmunoprecipitation experiments were done. Using
anti-PKCζ antibodies for immunoprecipitation, the EGFR was communoprecipitated. For control, we show that the use of IgG or the overexpression of the ΔCR1 loop mutant of EGFR leads to the loss of communoprecipitation with PKCζ. Interestingly, when the mutant EGFR-Y845F, which is missing the Src-specific phosphorylation site Tyr845 in EGFR (34), was cotransfected, we also found a clearly diminished communoprecipitation with PKCζ (Fig. 6A). Resting communoprecipitation may result from endogenously expressed EGFR.

Figure 6B shows that PKCζ tyrosine phosphorylation was indeed significantly affected by the Src-specific inhibitor PP2 (Fig. 6D, left) and completely abolished by coexpression of the dominant-negative c-Src mutant SrcRF (K295R/Y527F). Next, we further examined the role of the c-Src-specific phosphorylation site pY845 in EGFR. Initially, we cotransfected PKCζ with EGFR WT, the mutant EGFR-Y845F, and, for comparison, with several other EGFR mutants where major autophosphorylation sites have been replaced by phenylalanine. Only the coexpression of EGFR-Y845F resulted in a clearly decreased basal as well as EGF-induced tyrosine phosphorylation of PKCζ (Fig. 6C and D, right). Then, we performed kinase activity assays of immunoprecipitated PKCζ with MBP as
substrate. In general, when coexpressed with EGFR, a high basal activity of PKCζ is revealed, which may be further increased due to cell stimulation with EGF (Fig. 6E; see also Fig. 2D). When we coexpressed the EGFR-Y845F mutant instead of the WT, this effect of EGF was lost. Again, coexpression of another mutant, EGFR-Y1173F, with intact Y845, did not affect the stimulation of PKCζ kinase activity by EGF. In another approach, we synthesized a biotinylated phosphopeptide corresponding to the NH$_2$- and COOH-terminal flanking six amino acids of EGFR-pY845 (Fig. 6F). Pull-down experiments with cell lysates overexpressing PKCζ showed that the phosphopeptide bound significantly more PKCζ than did the nonphosphorylated form (Fig. 6G and H).

For control, tyrosine was replaced by alanine. When we used this peptide, we measured a binding of PKCζ closely corresponding to that obtained with the nonphosphorylated Y-peptide. For additional control, there was no significant difference between these peptides with respect to binding of overexpressed classic PKCα or novel PKCζ. These findings define the specificity of the binding of PKCζ to the phosphorylated tyrosine within the sequence.

**Discussion**

There is increasing evidence suggesting an important role of PKCζ in multiple signaling pathways that are critically involved in the regulation of tumorigenesis (15). Thus, in lung and breast cancer cells, PKCζ has been shown to support cell survival by Akt (24) and/or to abrogate the proapoptotic function of Bax (26). Furthermore, in several breast carcinoma cells (25) and non–small-cell lung cancer cells (35), PKCζ is required for the function of EGF-induced chemotaxis in the metastasis of cancer cells (36, 37). More recently, PKCζ was shown to mediate EGF-induced activation of ERK in several HNSCC cell lines (11). However, the molecular mechanism by which PKCζ is activated through EGF remains completely unclear. To elucidate this problem, we initially investigated the potency of EGF to induce tyrosine phosphorylation of PKCζ. Tyrosine phosphorylation of several PKC isoforms has been repeatedly suggested as an alternative mode of PKC activation (19, 20, 32, 38). Nevertheless, ligand-induced tyrosine phosphorylation of PKCζ has not yet been shown. Initially, we found tyrosine phosphorylation of PKCζ by EGF in several cancer cell lines with high levels of endogenously expressed PKCζ. Furthermore, to mimic tumorigenic conditions, we used COS-7 cells transiently transfected with EGFR and PKCζ. In this overexpressing model, we found a time- and concentration-dependent tyrosine phosphorylation of PKCζ due to activation of EGFR. Tyrosine phosphorylation of PKCζ essentially involved EGFR tyrosine kinase activity and resulted in catalytic activation of PKCζ. Furthermore, in the COS-7 cell model as well as in several carcinoma cell lines, we showed the critical involvement of PKCζ in the activation of ERK by EGF. Our results also confirm the finding of Cohen et al. (11) that in HNSCC cells, the requirement of PKCζ for maximal ERK activation by EGF seems to be cell specific. In addition, here we can show that not only in HNSCC but also in breast carcinoma cells, PKCζ may contribute to activation of ERK downstream of EGFR. However, rather than overexpression of EGFR, the prerequisite in these carcinoma cell lines seems to be a high expression level of PKCζ.

To address a putative connection of tyrosine phosphorylation of PKCζ with the activation of ERK, several experimental strategies have been chosen. First, we generated a novel mutant, PKCζ-Y417F, which lost three important functional properties compared with the WT PKCζ. This mutant (a) was not catalytically activated by EGF; (b) was not able to enhance activation of ERK induced by EGF; and (c) did not elevate EGF-mediated stimulation of DNA synthesis. The EGF-induced tyrosine phosphorylation of PKCζ-Y417F was significantly reduced but not completely abolished, suggesting that in response to EGF, multiple tyrosine residues of PKCζ are phosphorylated but only tyrosine 417 is critically involved in both the enzymatic activation of PKCζ and the PKCζ-mediated activation of ERK and, subsequently, stimulation of cell proliferation. Indeed, using mass spectrometry analysis of phosphopeptides, we identified at least three additional tyrosine residues in PKCζ that are phosphorylated in response to EGF.1 Furthermore, the catalytically incompetent mutant PKCζ-T410A, which exhibits the critical tyrosine in position 417, was compared with the novel mutant PKCζ-Y417F still exhibiting the residue T410, which is critical for the catalytic maturation of PKCζ by PDK1. Both mutants are neither catalytically activated by EGF nor capable of increasing the EGF-induced activation of ERK, but rather display a different pattern of tyrosine phosphorylation. Compared with the WT PKCζ, the basal (due to EGFR overexpression) tyrosine phosphorylation of the catalytically competent Y417F mutant is slightly diminished but that of the catalytically incompetent mutant T410A is considerably decreased. In contrast, both mutants reveal the same extent of reduction in tyrosine phosphorylation in response to EGF (Fig. 3D and E). This apparent discrepancy results from the lowered basal level of the T410A mutant, which is thereby the relatively stronger tyrosine phosphorylated by EGF than the Y417F mutant. The reason for this discrepancy is not yet understood, but conformational restrictions might be assumed to lead to different phosphorylation of multiple tyrosines. However, it may be concluded that only catalytically competent PKCζ becomes activated by EGF and that both T410 and Y417 are necessary for EGF-induced tyrosine phosphorylation and thereby activation of PKCζ.

Furthermore, whereas multiple tyrosine residues of PKCζ may be phosphorylated in response to EGF, the activation of PKCζ by EGF and the PKCζ-mediated stimulation of ERK activity are solely dependent on tyrosine in position 417. The catalytic domain of PKCζ contains

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1 H. Daub and C. Liebmann, unpublished results.
four tyrosine residues, 356, 374, 417, and 428, which are highly conserved among the PKC family. Based on phosphorylation probability analysis, the tyrosine residues in positions 417 and 428 in the activation loop have been predicted as potential phosphorylation sites, but finally, Y428 was selected as the favored phosphorylation site that might induce catalytic activation alternatively to Thr410 phosphorylation (21). Our data suggest the tyrosine residue 417, which is located closer to the activation loop, as a specific target of EGFR-mediated activation of PKCζ. It should be noted that tyrosine phosphorylation of PKCζ may not only result in catalytic activation but also provide docking sites for SH2 domain–containing proteins. Thereby, EGF might also induce a kinase-independent scaffold function of PKCζ. For example, the sequence PNPY417IAP within the structure of PKCζ might represent a potential consensus sequence for the COOH-terminal SH2 domain of phospholipase Cγ (39).

We also compared the EGF-induced activation of ERK in PKCζ-expressing Mef cells with that in PKCζ−/− Mef cells. The data obtained using increasing concentrations of EGF suggest that the EGF-induced activation of ERK was not completely dependent on the presence of PKCζ. Nevertheless, it becomes also evident that the presence of PKCζ is

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**FIGURE 6.** Critical role of the Src-specific site pY845-EGFR in mitogenic PKCζ signaling. A, PKCζ may physically associate with the activated EGFR. COS-7 cells were cotransfected with PKCζ and WT EGFR, the dimerization-inactive mutant EGFR-ΔCR1, or the mutant EGFR-Y845F. After serum starvation, the cells were stimulated with 10 nmol/L EGF for 1 minute. Lysates were prepared and immunoprecipitated with anti-PKCζ antibody or IgG (as negative control) and analyzed by Western blotting for coimmunoprecipitation of EGFR. Representative results of two independent experiments. B, COS-7 cells transiently cotransfected with PKCζ and EGFR were serum starved, pretreated with 50 nmol/L PP-2 or vehicle for 30 minutes, and then treated with 10 nmol/L EGF for 5 minutes. As indicated, in one set of these experiments, dominant-negative Src (Src-RF) was additionally cotransfected. Immunoprecipitations and Western blotting were done as indicated. Representative results of three separate experiments. C, COS-7 cells were cotransfected with PKCζ and either WT EGFR or the different EGFR mutants as indicated, serum starved overnight, treated with 10 nmol/L EGF for 5 minutes, and lysed; PKCζ was immunoprecipitated and analyzed for tyrosine phosphorylation as described. The protein expression of the EGFR constructs was determined by Western blotting with anti-EGFR antibody (13G8). Representative blots are shown. D, data quantification (from B and C). Columns, means; bars, SD. Left: *, significantly higher (EGF versus basal); #, significantly lower (EGF and PP-2 versus EGF), compared with the vehicle-treated controls (P < 0.05). Right: *, significantly higher compared with the basal value; +, significantly lower compared with the EGFR WT (P < 0.05). The data are from three to four separate experiments.
necessary for the full activation of ERK in response to EGF. It should be noted that the ERK activation in PKCζ−/− Mef cells sometimes varies between individual experiments. This might explain why in previous studies no differences in ERK activation were found between WT and PKCζ−/− cells (33).

In this study, we also investigated how the EGFR might mediate tyrosine phosphorylation and catalytic activation of PKCζ. Our findings indicate that the EGFR tyrosine kinase is essentially required. Thus, PKCζ physically associates with the EGFR; the tyrosine phosphorylation is dependent on EGFR dimerization; and the EGFR-specific tyrosine kinase inhibitor AG1478 completely prevents PKCζ tyrosine phosphorylation. Additionally, the significant inhibition of PKCζ tyrosine phosphorylation by the Src-inhibitor PP2 and the complete prevention of PKCζ tyrosine phosphorylation by dominant-negative c-Src suggest the critical involvement of c-Src. Several lines of evidence strongly indicate the dependency of PKCζ tyrosine phosphorylation on the unique tyrosine residue 845 of EGFR, which is solely phosphorylated through c-Src. Compared with the WT, (a) the immunoprecipitation of PKCζ with the mutant EGFR-Y845F is decreased; (b) the tyrosine phosphorylation of PKCζ is clearly diminished when the mutant EGFR-Y845F is coexpressed; (c) the catalytic activation of PKCζ by EGF is reduced in the presence of the mutant EGFR-Y845F; and (d) the phosphopeptide GAEEKEpYHAEGGK-NH₂, which corresponds to the surrounding sequence of pY845-EGFR, binds significantly more PKCζ in vitro compared with the nonphosphorylated peptide. Previously, two downstream effectors of pY845-EGFR, STAT5b (40) and the cytochrome c oxidase subunit II (41), have been reported. We recently showed the importance of pY845-EGFR for the interaction of EGFR with PKCε and postulated a critical role of the C2-domain of PKCζ in the recognition of pY845-EGFR (42). Here we show a novel importance of pY845-EGFR as a putative docking site for the aPKCζ, which contains a PB1 domain instead of a C2 domain at the NH₂ terminus. The PB1 (Phox and Bem1) domain is composed of about 80 amino acid residues mediating protein-protein interactions. These contacts are based on electrostatic interactions between conserved basic cluster residues located on one side of a PB1 domain and conserved acidic residues in the so-called OPCA motif of another PB1 domain (43). In that way, the ability of aPKCs has been shown to form protein modules (e.g., with ZIP/p62 or with MAP/ERK kinase 5; ref. 10). The surrounding of EGFR-Y845 is dominated by four acidic glutamic acid residues (E841, E842, E844, and E848). Furthermore, phosphorylation of the tyrosine residue may increase the potential for electrostatic interactions (44). PKCζ contains two conserved lysine residues with clustering basic arginine residues, such as K19 behind R15 and R17 and K 112 followed by R116, R117, R120, R121, and R123. Lysine in position 112 is adjacent to the PB1 domain in PKCζ but corresponds to K355 in

![FIGURE 6. Continued.](image)

E, COS-7 cells were cotransfected with PKCζ and WT EGFR, EGFR-Y845F, or EGFR-Y1173F. Serum-starved cells were stimulated with 10 nmol/L EGF for 5 minutes. PKCζ immunoprecipitates were used for the immune complex kinase assay with MBP as substrate as described in Materials and Methods. MBP phosphorylation was measured by autoradiography and the amount of immunoprecipitated PKCζ by reblotting with anti-PKCζ antibody. EGFR expression was determined by Western blotting using aliquots of the cell lysates. The experiments were repeated twice with similar results. F, structure of the biotinylated phosphopeptide derived from the Y845-containing sequence of EGFR. G, COS-7 cells were transiently transfected with cDNA of PKCζ or, for comparison, PKCa or PKCc. Lysates (corresponding to 1 mg protein) were subjected to pull-down assays using the biotinylated phosphopeptide (pY-peptide), the nonphosphorylated sequence (Y-peptide), or a control peptide where tyrosine was replaced by alanine (A-peptide; 10 μg per assay) and streptavidin-agarose beads. Precipitates were subjected to SDS-PAGE and analyzed by Western blotting with the respective PKC-specific antibodies. In the rows below the pull-down blots, the expression levels of the different PKC isoforms are shown. H, quantitative analysis of the increase in PKCζ pull-down. Columns, means; bars, SD. *P < 0.05, significantly higher. Results from six separate experiments.
p67phox, which plays a critical role in the P81 domain interaction of p40phox/p67phox (43). It may be speculated that the pY845-dependent association of EGFR and PKCζ may be mediated by electrostatic interactions between the acidic glutamic acid cluster surrounding Y845 and one of the basic amino acid clusters in PKCζ, which may be additionally enhanced by the phosphorylation of EGFR-Y845. This hypothesis is also supported by our finding that in the breast carcinoma cell line MDA-MB-453, which lacks EGFR but overexpresses ErbB2, the basal tyrosine phosphorylation of PKCζ is extremely high but not further enhanced by EGF treatment (Fig. 1A). An explanation may be provided by the sequence flanking Y877 in ErbB2, which corresponds to Y845 in EGFR. Compared with the four negative charged residues in ErbB1, the corresponding ErbB2 sequence contains five acidic amino acids, three aspartic acid residues (D871, D873, and D879), and two glutamic acid residues (E874 and E876) and, therefore, has a higher basal electrostatic potential to bind PKCζ than does the EGFR. Detailed investigations will be necessary to verify this hypothesis. Taken together, our results suggest that in cells overexpressing PKCζ, such as in several carcinoma cell lines, EGF may regulate activation of ERK by dual mechanisms. In a particular cellular context, the classic Ras-dependent ERK pathway may be assisted by an additional PKCζ-mediated pathway to ERK. Phosphorylation of the unique tyrosine residue 845 leads to enhanced recruitment of PKCζ to the EGFR. Then, EGFR-associated PKCζ becomes tyrosine phosphorylated through EGFR tyrosine kinase and/or c-Src and thereby catalytically activated. Subsequently, PKCζ may contribute to elevated ERK activation. We pos-

ulate that this pathway occurs cell-specifically and depends on the expression pattern of PKCζ and the interacting cellular network. It does not represent a principal mechanism valid for all cell types. However, our results support a key role of PKCζ in several aggressive tumors and highlight the suppression of EGFR-PKCζ interaction as a novel therapeutic strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Sarah Parsons (University of Virginia, Charlottesville, VA) for the expression plasmid encoding the EGFR-Y845F mutant; Dr. F.D. Boehmer (University of Jena, Jena, Germany) for the cDNAs encoding the human EGFR WT and the EGFR autophosphorylation site mutants; Dr. Joan Brugge (Harvard Medical School, Boston, MA) for the plasmid encoding the dominant-negative mutant c-SrcRF (K295R/Y527F); Dr. A.W. Burgess (Ludwig Institute for Cancer Research, Melbourne, Australia) for the EGFR-ΔΔCR1 (ΔΔ42-259) construct; Dr. K. Pfitzenmaier (Institute of Cell Biology and Immunology, Stuttgart, Germany) for PKCζ-T410A-EYFP-N1; Dr. M. Leitges (Biotechnology Centre of Oslo, Oslo, Norway) for PKCζ-mutant c-Src and Mel WT cells; and Dr. Alexander Berndt (Institute of Pathology, University of Jena, Jena, Germany) for the HNSCC and MCF7 cell lines.

Grant Support

Deutsche Forschungsgemeinschaft grant SFB 604/A5 (C. Liebmann).

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Received 04/20/2009; revised 03/09/2010; accepted 03/10/2010; published OnlineFirst 04/20/2010.

References

Molecular Cancer Research

Activation by Tyrosine Phosphorylation as a Prerequisite for Protein Kinase Cζ to Mediate Epidermal Growth Factor Receptor Signaling to ERK

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Mol Cancer Res 2010;8:783-797. Published OnlineFirst April 20, 2010.

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