

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

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Abstract

The atypical protein kinase C ζ (PKC ζ) 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 PKC ζ in several HNSCC cells, breast carcinoma cells, as well as mouse embryonic fibroblasts. In COS-7 cells overexpressing EGF receptor (EGFR) and PKC ζ as a tumor cell model, we show that PKC ζ tyrosine phosphorylation by EGF is induced by catalytic activation. Using a loss-of-function mutant of PKC ζ , we can show that the tyrosine residue 417 in PKC ζ plays an important role in both PKC ζ activation and the ability of PKC ζ to mediate activation of ERK. The importance of PKC ζ in EGF-induced ERK activation can also be shown in several HNSCC and breast carcinoma cell lines as well as in PKC ζ -deficient mouse embryonic fibroblasts. In addition, we present several lines of evidence suggesting the physical association of PKC ζ 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 PKC ζ . This study characterizes PKC ζ as a novel mitogenic downstream mediator of EGFR and indicates PKC ζ 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 up-regulated, 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 C γ 1 (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 C ζ (PKC ζ) 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 PKC ζ as well as by other PKC isoforms has been repeatedly described (12, 13), the molecular mechanism of how PKC ζ 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

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doi: 10.1158/1541-7786.MCR-09-0164

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(α , β , γ); the Ca^{2+} -independent and diacylglycerol-dependent (novel) nPKCs (δ , ϵ , η , θ); and the (atypical) aPKCs (λ , ζ), which are also Ca^{2+} independent but do not require diacylglycerol for activation (14). These atypical PKCs reveal an atypical NH_2 -terminal structure and a relatively low sequence homology with the other PKC isoforms. Thus, PKC ζ consists of four functional domains and motifs, including a PB1 domain in the NH_2 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. PKC ζ 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 PKC ζ activation (16). The initial phosphorylation at Thr⁴¹⁰ is mediated by phosphoinositide-dependent kinase 1 (PDK1) through a pathway including the activation of phosphoinositide 3-kinase and the formation of phosphatidylinositol 3,4,5-trisphosphate, which activates PDK1. Alternatively, phosphatidylinositol 3,4,5-trisphosphate can also directly interact with PKC ζ and release the PS-dependent autoinhibition. Obviously, both effects of phosphatidylinositol 3,4,5-trisphosphate are necessary for the full activation of PKC ζ (15, 17). Recently, phospholipase D2 has been identified to interact with PKC ζ through protein-protein interaction leading to both direct activation of PKC ζ and an increase in activation loop phosphorylation (18). Alternatively, tyrosine phosphorylation might be another, but less well-characterized, mode of PKC activation and was mainly reported for PKC δ (19) and PKC α (20). Thus, the *in vitro* tyrosine phosphorylation of recombinant PKC ζ has been shown. Based on phosphorylation probability analysis as well as homology structural modeling, the Tyr⁴²⁸ in PKC ζ in the catalytic loop was predicted as the site to be phosphorylated (21).

Like EGFR, PKC ζ 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 PKC ζ /EGFR overexpression strategy as an approach to investigate a putative role of PKC ζ 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 PKC ζ . Using mouse embryonic fibroblast (Mef) cells deficient in PKC ζ and a loss-of-function mutant, PKC ζ -Y417F, we can show that tyrosine phosphorylation and thereby catalytic activation of PKC ζ 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 PKC ζ . Thus, our results define PKC ζ as a

novel downstream target of EGFR and provide some mechanistic explanation on the importance of PKC ζ 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 PKC ζ (PKC ζ ^{-/-} 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 PKC ζ , PKC α , 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. EGFR antibody (clone 13G8) was purchased from NanoTools Antikörpertechnik. The monoclonal EGFR 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 PKC ζ and PKC α have been described previously (27). PKC ζ -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- Δ CR1 (Δ 242-259) 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 $\mu\text{g}/\text{mL}$ streptomycin sulfate, and 0.25 $\mu\text{g}/\text{mL}$ 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

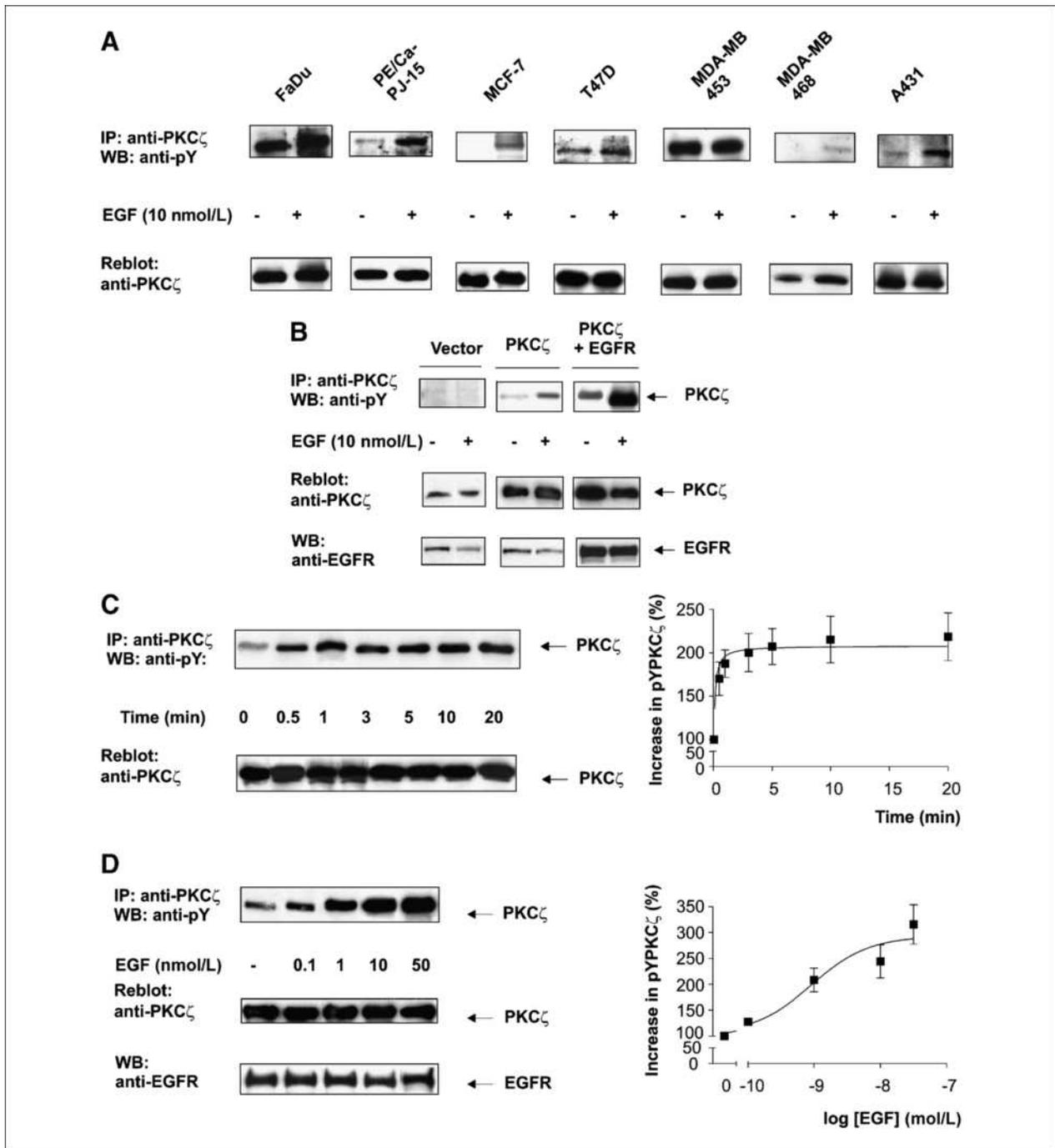


FIGURE 1. Characterization of EGF-induced tyrosine phosphorylation of PKC ζ . **A**, EGF-induced tyrosine phosphorylation of PKC ζ in various carcinoma cell lines. Serum-starved cells were stimulated with 10 nmol/L EGF for 5 minutes. PKC ζ was immunoprecipitated, analyzed by Western blotting with anti-phosphotyrosine antibody (4G10), and reprobbed with anti-PKC ζ antibody. For each lane, 100 μ g 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 PKC ζ or cotransfected with PKC ζ and EGFR. After serum starvation overnight, cells were stimulated with 10 nmol/L EGF for 5 minutes. PKC ζ was immunoprecipitated (IP) and analyzed by Western blotting (WB) with anti-phosphotyrosine antibody and reprobbed with anti-PKC ζ 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 PKC ζ in COS-7 cells. Only PKC ζ in **C** and PKC ζ 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 PKC ζ tyrosine phosphorylation in response to EGF compared with nonstimulated cells; bars, SE. The data have been normalized to the levels of PKC ζ immunoprecipitates.

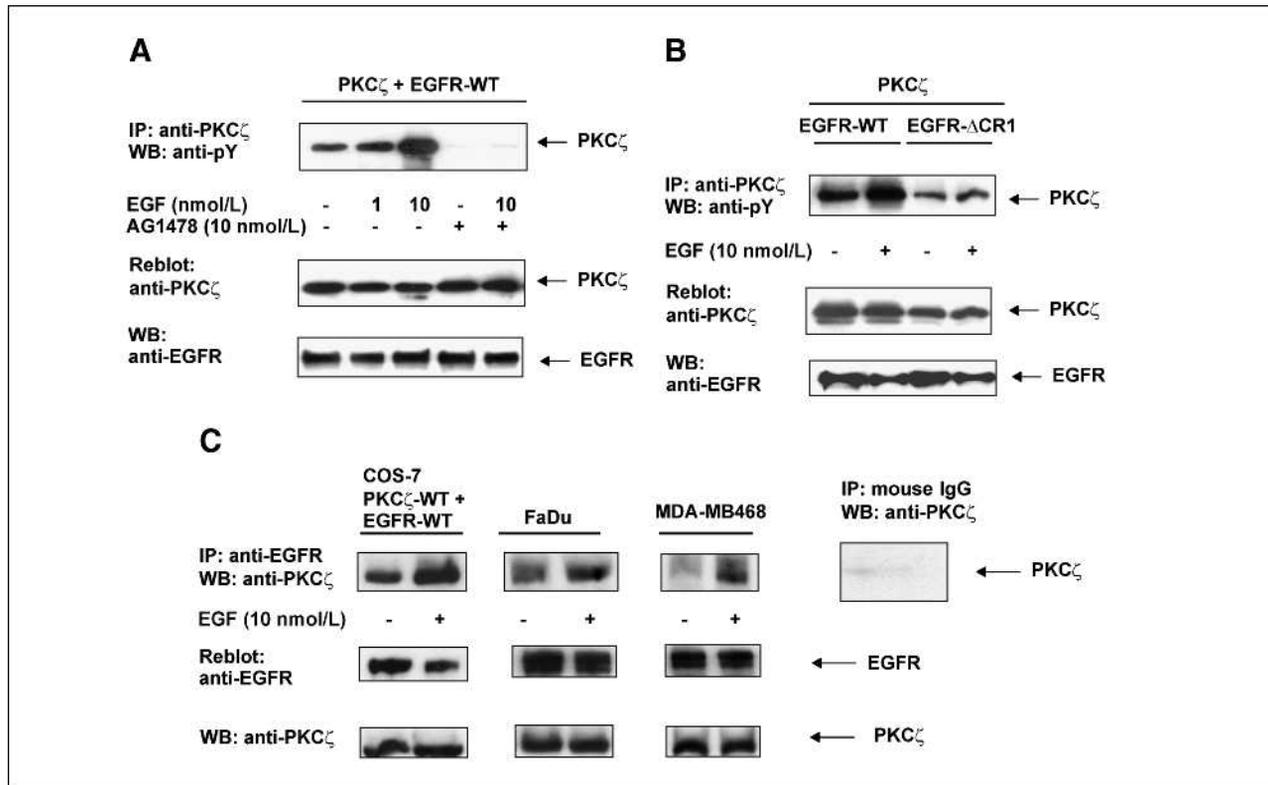


FIGURE 2. EGF-induced and EGFR-mediated catalytic activation of PKC ζ . **A**, COS-7 cells transiently cotransfected with PKC ζ and EGFR were serum starved, pretreated with 10 nmol/L AG1478 or vehicle for 30 minutes, and then stimulated with 1 or 10 nmol/L EGF for 5 minutes. Immunoprecipitation and Western blotting were done as described. Aliquots were used to determine the respective expression levels by Western blotting. Representative results of three separate experiments. **B**, COS-7 cells cotransfected with PKC ζ and either WT EGFR or the dimerization-inactive mutant EGFR- Δ CR1 were serum starved, treated with 10 nmol/L EGF for 5 minutes, lysed, immunoprecipitated with anti-PKC ζ antibody, analyzed by Western blotting with anti-phosphotyrosine antibody (4G10), and reprobed with anti-PKC ζ antibody. The equality of EGFR expression was controlled by Western blotting with anti-EGFR antibody (13G8). Representative results of two separate experiments. **C**, PKC ζ may physically associate with the activated EGFR. COS-7 cells were cotransfected with PKC ζ and EGFR; FaDu cells or MDA-MB-468 cells were serum starved and then stimulated with 10 nmol/L EGF for 1 minute. Lysates were prepared and immunoprecipitated with anti-EGFR antibody or IgG (as negative control) and analyzed by Western blotting for coimmunoprecipitation of PKC ζ . Representative results of three independent experiments.

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 μ g/mL aprotinin, and 20 μ g/mL leupeptin. Lysates were centrifuged at 14,000 $\times g$ 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 phosphorimager. 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 Bn-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 PKC ζ or PKC α were lysed. Equal amounts of protein from each sample were incubated overnight with 10 μ g of the respective biotinylated peptides at 4°C. Then, 20 μ L 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

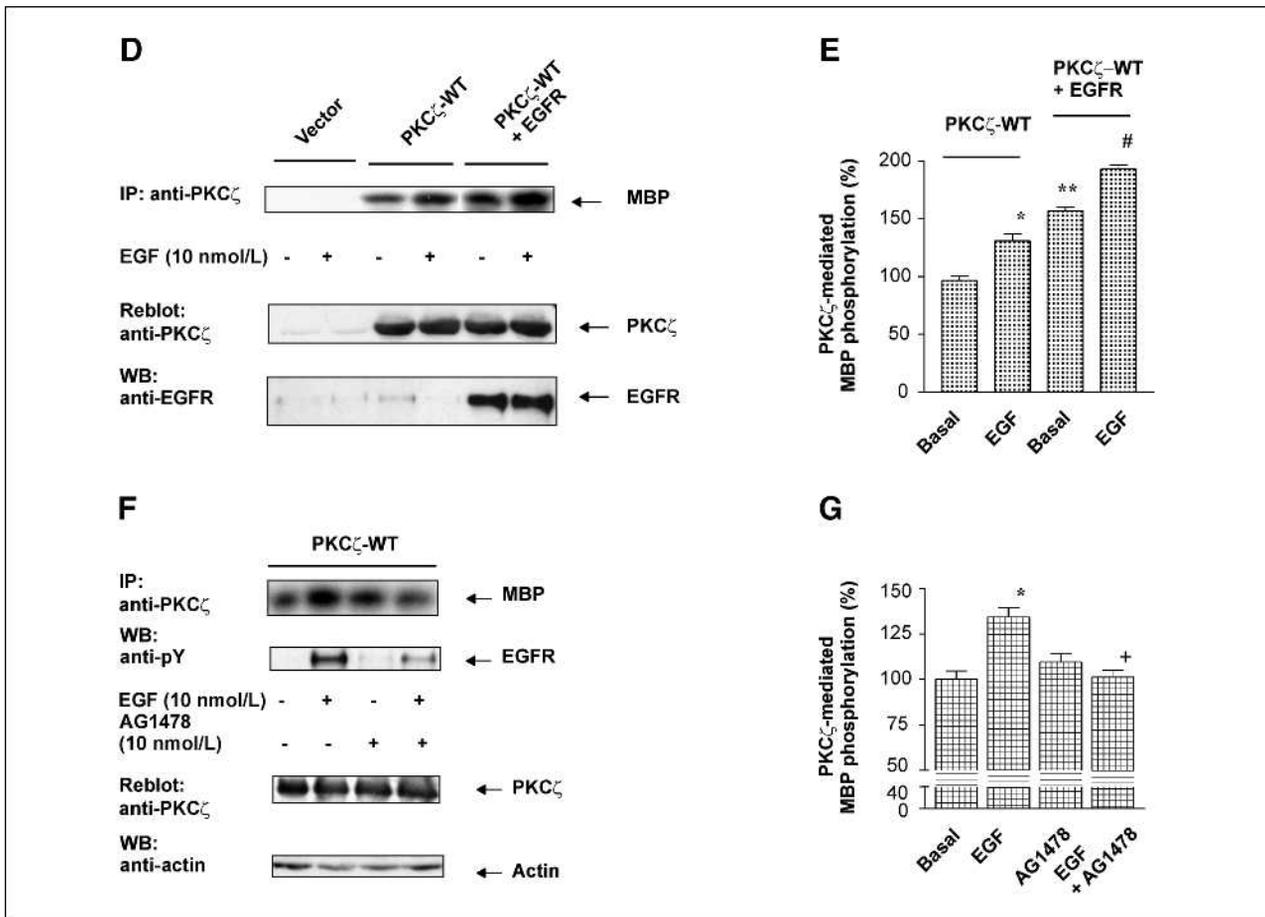


FIGURE 2. Continued. D, COS-7 cells cotransfected with EGFR and PKC ζ as indicated were serum starved and stimulated with 10 nmol/L EGF for 5 minutes. PKC ζ immunoprecipitates were assayed in the presence of [γ - 32 P]ATP for PKC activity using MBP as substrate. Top, an autoradiogram of MBP phosphorylation, which is representative of six independent experiments. Membranes were probed with anti-PKC ζ antibody. Using aliquots of the lysates, the equal expression of EGFR was determined by Western blotting. E, quantified data from D, normalized to the PKC ζ immunoprecipitates. The basal activity due to overexpression of PKC ζ (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 PKC ζ 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 PKC ζ 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 PKC ζ . Representative results of four independent experiments. G, quantification of the data in F normalized to the PKC ζ immunoprecipitates. Columns, means; bars, SD. *, significantly higher compared with basal; +, significantly lower compared with EGF ($P < 0.05$).

in SDS sample buffer for 5 minutes. Proteins were analyzed by Western blotting using anti-PKC ζ or anti-PKC α antibodies as indicated. The protein content was measured using the Roti-Nanoquant kit according to the manufacturer's protocol.

Site-directed mutagenesis. PKC ζ -Y417F was generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the WT human PKC ζ as a template. A pair of primers (35-mers) fully complementary to each other was designed to contain the mutation -Y417Ff (sense strand, 5'-ctgcggaaccccgaaattcatcgccccgaaatcc-3') and Y417Fr (antisense strand, 5'-ggatttcggggcgatgaaattcgggggtccgcag-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.

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

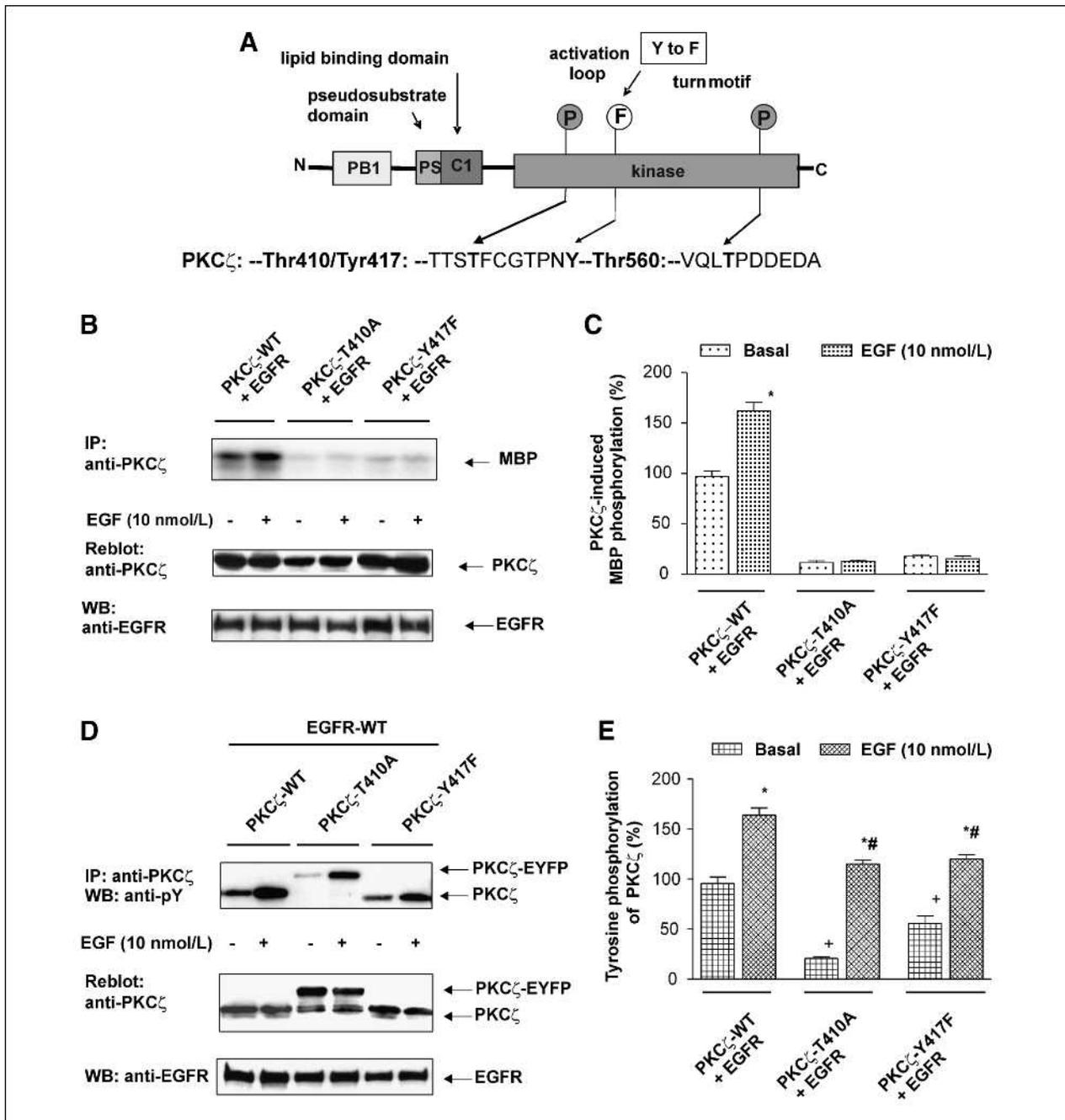


FIGURE 3. The mutant PKC ζ -Y417F reveals reduced tyrosine phosphorylation and is insensitive toward EGF-induced catalytic activation.

A, schematic depiction of the domain organization of PKC ζ . 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 PKC ζ , the catalytically inactive mutant PKC ζ -T410A (as negative control), or the novel mutant PKC ζ -Y417F. The cells were serum starved and stimulated with 10 nmol/L EGF for 5 minutes. The PKC ζ immunoprecipitates were assayed in the presence of [γ - 32 P]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-PKC ζ 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 PKC ζ (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 PKC ζ . D, COS-7 cells were cotransfected with EGFR and the PKC ζ 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 reprobbed with anti-PKC ζ antibody. Representative of six experiments. E, quantitative analysis of PKC ζ tyrosine phosphorylation (data from D). Both basal and EGF-induced tyrosine phosphorylation of the mutants are compared with those of the WT PKC ζ (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 PKC ζ reblots.

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 μ Ci 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 PKC ζ .

To investigate the molecular mechanism by which PKC ζ might be able to mediate oncogenic EGFR signaling, we considered recently published data suggesting a critical role of PKC ζ in EGF-induced activation of MAPK in HNSCC (11) or chemotaxis in breast carcinoma (25) cells. We used these carcinoma cell types to investigate PKC ζ tyrosine phosphorylation, and after treating the cells with 10 nmol/L EGF for 5 minutes, we found significant tyrosine phosphorylation of PKC ζ 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 but expresses a high level of ErbB2 (30), revealed an extremely high basal tyrosine phosphorylation of PKC ζ (Fig. 1A). Next, we studied PKC ζ tyrosine phosphorylation in COS-7 cells used as a transfection model. In these cells, due to low levels of endogenous PKC ζ tyrosine phosphorylation by EGF was not detectable. In contrast, when we mimicked the conditions occurring in the majority of carcinoma cells by overexpressing PKC ζ and EGFR, significant PKC ζ tyrosine phosphorylation was detectable. When PKC ζ and EGFR were cotransfected, we obtained an approximately 3.5-fold increase in PKC ζ 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 PKC ζ tyrosine phosphorylation was stable up to 20 minutes (Fig. 1C). Furthermore, the effect of EGF was concentration dependent and revealed an EC₅₀ value of ~0.4 nmol/L (Fig. 1D).

EGFR-mediated tyrosine phosphorylation of PKC ζ leads to catalytic activation of PKC ζ . The EGF-induced tyrosine phosphorylation of PKC ζ was completely prevented by the EGFR tyrosine kinase-specific inhibitor AG1478 (Fig. 2A). Furthermore, when the nondimerizing Δ CR1 loop EGFR mutant (31) was overexpressed, PKC ζ was not tyrosine phosphorylated by EGF (Fig. 2B). Coim-

munoprecipitation studies were done to address the question whether tyrosine phosphorylation of PKC ζ requires the direct interaction with the EGFR. The basal as well as EGF-induced association between PKC ζ 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 PKC ζ tyrosine phosphorylation. Whereas under endogenous conditions there was no detectable activation of PKC ζ , overexpression of PKC ζ 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 PKC ζ was prevented by AG1478, suggesting the critical involvement of EGFR tyrosine kinase in tyrosine phosphorylation as well as activation of PKC ζ by EGF (Fig. 2F and G).

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

Stimulation with EGF leads to the catalytic activation of WT PKC ζ (Fig. 3B and C; see also Fig. 2). As a negative control, we used the catalytically inactive mutant PKC ζ -T410A, which was available as an EYFP-tagged construct. The tag had no importance for the present investigation. Surprisingly, the novel mutant PKC ζ -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 PKC ζ , 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 PKC ζ . 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 PKC ζ -induced activation of ERK. In COS-7 cells overexpressing EGFR, EGF-induced activation of MAPK (ERK1/2) was significantly elevated when PKC ζ was additionally transfected. This effect was not observed when the

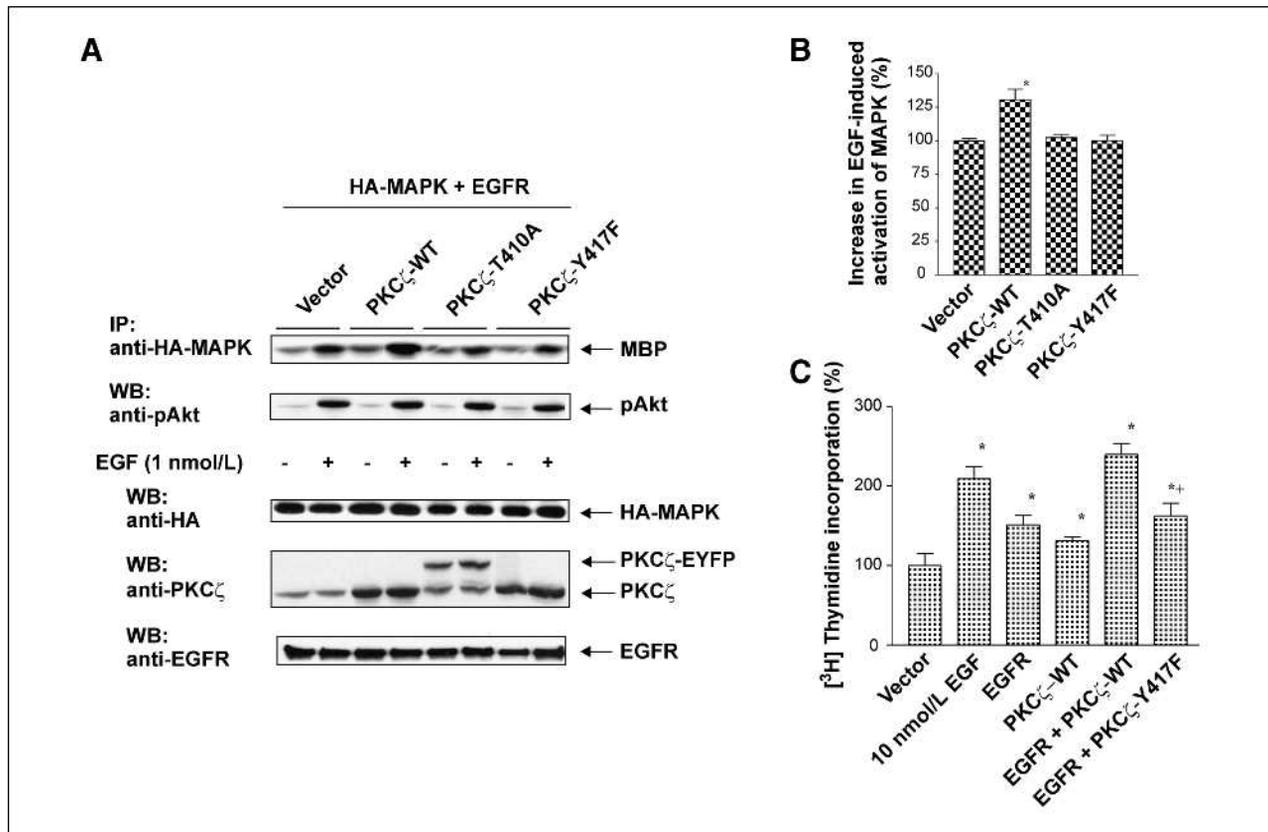


FIGURE 4. PKC ζ activated by tyrosine phosphorylation is involved in EGFR-mediated activation of ERK. **A**, COS-7 cells were transiently cotransfected with HA-ERK2 and EGFR as well as empty vector, WT PKC ζ , PKC ζ -T410A-EYFP, or PKC ζ -Y417F. Serum-starved cells were stimulated with 1 nmol/L EGF for 5 minutes. MAPK activity assay was done using anti-HA immunoprecipitates of cell lysates and MBP as substrate. MBP phosphorylation was detected by autoradiography. The membranes were probed with anti-HA antibody. Aliquots of the lysates were analyzed by Western blotting for the expression levels of PKC ζ and EGFR. **B**, data quantification (normalized to HA-MAPK). Columns, means; bars, SD. *, $P < 0.05$. The EGF-induced increase in ERK activation in the presence of WT or mutant PKC ζ is expressed as percentage and compared with vector-transfected cells. Results are from four separate experiments in duplicates. **C**, COS-7 cells were transfected in 24-well plates with the constructs as indicated using Lipofectamine 2000. Incorporation of [³H]thymidine was determined as described in Materials and Methods. Results are expressed as [³H]thymidine incorporated as percentage compared with vector-expressing cells. Columns, means; bars, SD. *, significantly higher compared with the respective basal values; +, significantly lower compared with the cells overexpressing the WT PKC ζ ($P < 0.05$). The data are obtained from three separate experiments in quadruplicates.

WT was replaced by the catalytically inactive mutants PKC ζ -T410A or PKC ζ -Y417F (Fig. 4A and B). Additionally, we estimated the influence of PKC ζ 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 [³H]thymidine incorporation can also be observed due to overexpression of either EGFR or WT PKC ζ . 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 ζ results in an additive effect on [³H]thymidine incorporation. In contrast, coexpression of the PKC ζ -Y417F mutant reduced DNA synthesis to the level of cells simply overexpressing EGFR. These findings suggest that on the EGFR module, PKC ζ 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 ζ 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 ζ inhibitor Myr-PS. In three of the cell lines (FaDu, MDA-MB 468, and MCF-7) also revealing tyrosine phosphorylation of PKC ζ (Fig. 1A), inhibition of PKC ζ 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 ζ or the catalytically inactive mutant PKC ζ -T410A or PKC ζ -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 ζ -WT results in additive ERK activation in response

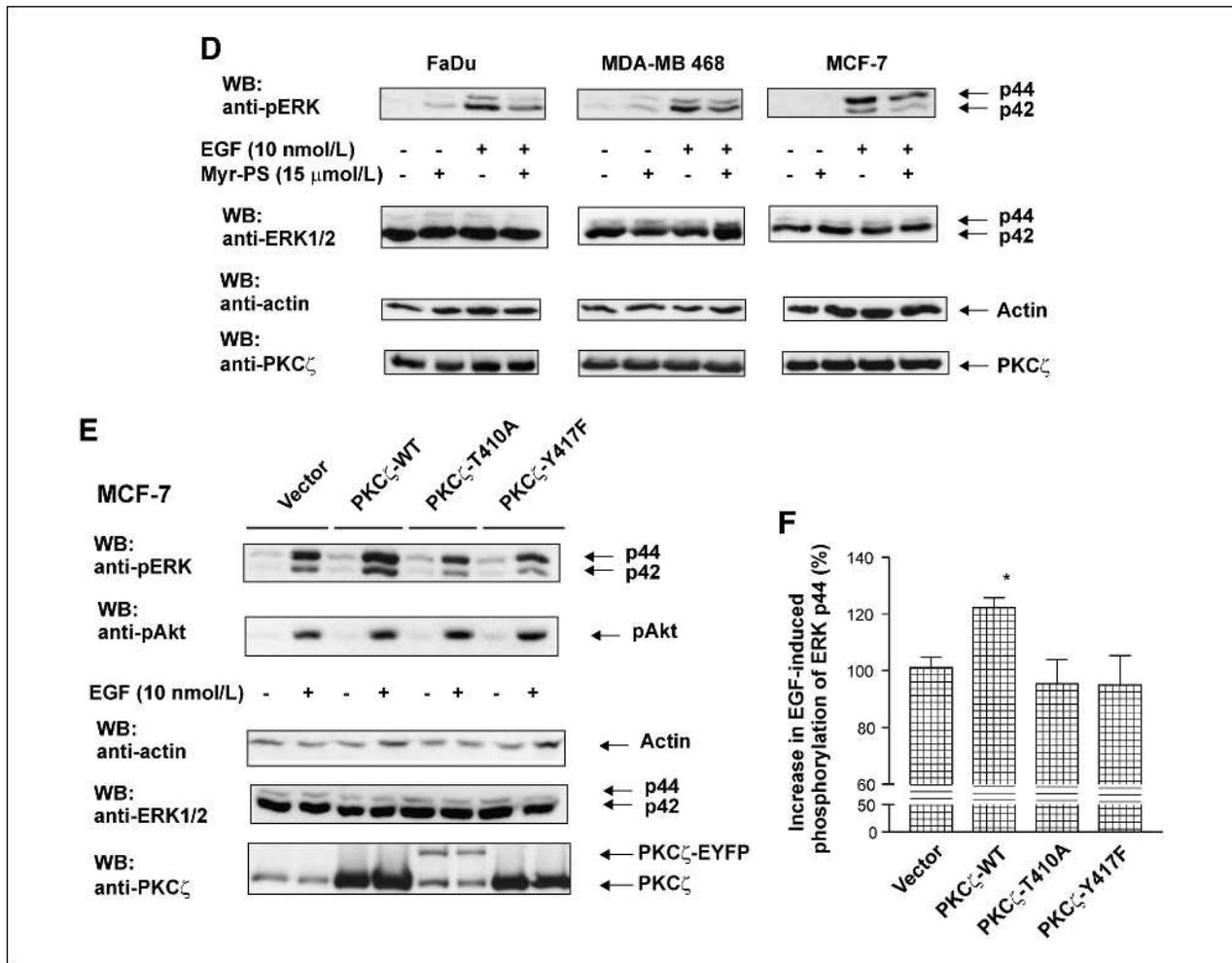


FIGURE 4. Continued. D, PKC ζ and ERK activation in carcinoma cell lines. Decrease of EGF-induced activation of ERK by the specific PKC ζ inhibitor Myr-PS (myristoylated PKC ζ pseudosubstrate) in FaDu, MDA-MB 468, and MCF-7 cells. After serum starvation, cells were preincubated with 15 μmol/L Myr-PS for 30 minutes and then stimulated with 10 nmol/L EGF for 5 minutes. Lysates were prepared as described and analyzed by Western blotting with anti-pERK antibody. Aliquots of these samples were run for Western blotting with mixed ERK1 and ERK2 antibodies. Immunoblotting with anti-actin was done for loading control and with anti-PKC ζ to show the high endogenous expression level of PKC ζ in these cell lines. Representative results of three separate experiments. E, EGF-induced ERK activation in MCF-7 cells overexpressing the inactive mutants compared with those overexpressing the WT PKC ζ . Using Lipofectamine 2000, MCF-7 cells were transiently transfected with WT PKC ζ , PKC ζ -T410A-EYFP, PKC ζ -Y417F, or vector. Serum-starved cells were stimulated with 10 nmol/L EGF for 5 minutes. Aliquots of the lysates were analyzed by Western blotting for ERK and Akt phosphorylation. For control, aliquots of these samples were separately run for Western blotting with mixed ERK1 and ERK2 antibodies. Actin was measured as loading control and expression of the PKC ζ constructs was determined. F, columns, means; bars, SD. *, $P < 0.05$, significantly higher compared with the vector. The data have been normalized to actin and are the mean from four separate experiments.

to EGF. In contrast, this additional effect did not occur when the catalytically inactive mutants were overexpressed.

ERK activation by EGF is reduced in PKC ζ ^{-/-} Mef cells. Next, we stimulated WT Mef cells expressing PKC ζ with increasing concentrations of EGF. We found a significant and dose-dependent increase in tyrosine phosphorylation of PKC ζ , revealing an EC₅₀ value of 0.06 nmol/L (Fig. 5A and B). To determine whether the lack of PKC ζ impairs ERK activation, both WT and PKC ζ -deficient (PKC ζ ^{-/-}) 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 PKC ζ , the EGF-

induced activation of ERK was significantly diminished compared with the WT Mef cells expressing PKC ζ . It should be noted that the basal activity of ERK is also slightly higher in WT cells than in PKC ζ ^{-/-} cells. Both the WT and the PKC ζ ^{-/-} Mef cells have been previously characterized in detail (33).

Role of the Src-specific phosphorylation site EGFR-Y845 in EGFR-PKC ζ association, EGF-induced tyrosine phosphorylation, as well as catalytic activation of PKC ζ . To further elucidate the mechanism of how the EGFR may mediate tyrosine phosphorylation of PKC ζ , additional coimmunoprecipitation experiments were done. Using

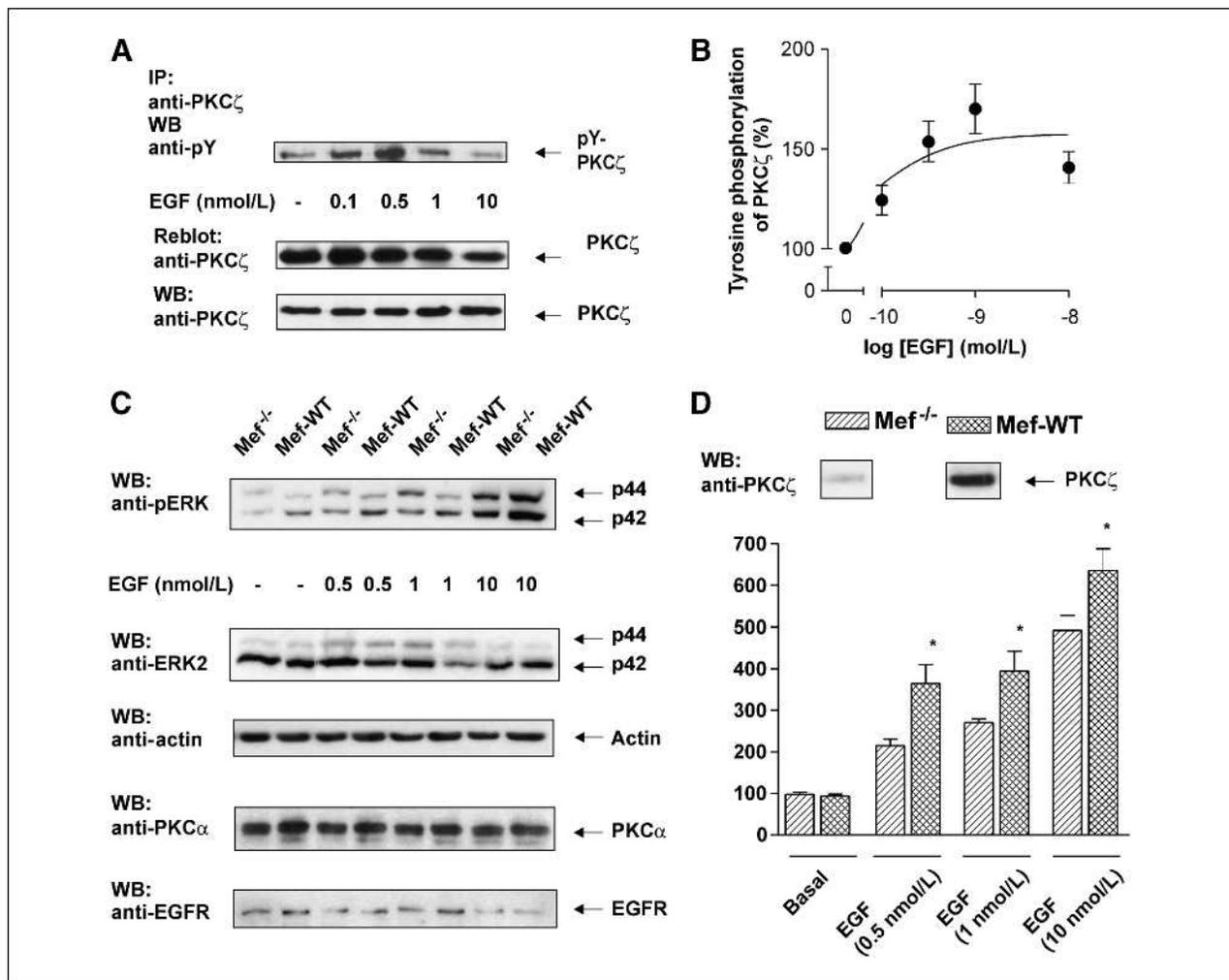


FIGURE 5. PKC ζ is tyrosine phosphorylated by EGF and contributes to EGF-induced activation of ERK in Mef cells. **A**, WT Mef cells expressing PKC ζ (see **C**) were serum starved and stimulated with increasing concentrations of EGF for 5 minutes, lysed, immunoprecipitated with anti-PKC ζ antibody, probed with anti-phosphotyrosine antibody (4G10), and reprobbed with anti-PKC ζ antibody. Shown is one of six independent experiments with similar results. **B**, dose-response curve of PKC ζ tyrosine phosphorylation in Mef cells treated with EGF. Points, mean from six separate experiments, normalized to the PKC ζ reblot; bars, SE. **C**, serum-starved WT or PKC ζ ^{-/-} Mef cells were stimulated with different concentrations of EGF for 5 minutes. Aliquots of the cell lysates (60 μ g protein each) were analyzed for activation of ERK by Western blotting with phosphosite-specific anti-pERK (Tyr²⁰⁴) antibody. Equal loading was controlled by measuring the contents of β -actin and PKC α per sample using the respective antibodies. For control, equal aliquots of these samples were run for Western blotting with mixed ERK1 and ERK2 antibodies. Representative blots of six independent experiments. **D**, the quantified data from **C** of site-specific phosphorylation (activation) of ERK in response to EGF have been normalized to actin and are expressed as percentage related to the respective basal values. Insets, expression levels of PKC ζ in the respective Mef cells. Columns, means; bars, SD. *, $P < 0.05$, significantly higher compared with PKC ζ ^{-/-} Mef cells.

anti-PKC ζ antibodies for immunoprecipitation, the EGFR was coimmunoprecipitated. For control, we show that the use of IgG or the overexpression of the Δ CR1 loop mutant of EGFR leads to the loss of coimmunoprecipitation with PKC ζ . 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 coimmunoprecipitation with PKC ζ (Fig. 6A). Resting coimmunoprecipitation may result from endogenously expressed EGFR.

Figure 6B shows that PKC ζ tyrosine phosphorylation was indeed significantly affected by the Src-specific inhib-

itor PP2 (Fig. 6D, left) and completely abolished by co-expression 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 PKC ζ 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 co-expression of EGFR-Y845F resulted in a clearly decreased basal as well as EGF-induced tyrosine phosphorylation of PKC ζ (Fig. 6C and D, right). Then, we performed kinase activity assays of immunoprecipitated PKC ζ with MBP as

substrate. In general, when coexpressed with EGFR, a high basal activity of PKC ζ 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 PKC ζ kinase activity by EGF. In another approach, we synthesized a biotinylated phosphopeptide corresponding to the NH₂- and COOH-terminal flanking six amino acids of EGFR-pY845 (Fig. 6F). Pull-down experiments with cell lysates overexpressing PKC ζ showed that the phosphopeptide bound significantly more PKC ζ 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 PKC ζ 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 PKC α or novel PKC ϵ . These findings define the specificity of the binding of PKC ζ to the phosphorylated tyrosine within the sequence.

Discussion

There is increasing evidence suggesting an important role of PKC ζ in multiple signaling pathways that are critically involved in the regulation of tumorigenesis (15). Thus, in lung and breast cancer cells, PKC ζ 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), PKC ζ is required for the function of EGF-induced chemotaxis in the metastasis of cancer cells (36, 37). More recently, PKC ζ was shown to mediate EGF-induced activation of ERK in several HNSCC cell lines (11). However, the molecular mechanism by which PKC ζ becomes activated through the EGFR remained completely unclear. To elucidate this problem, we initially investigated the potency of EGF to induce tyrosine phosphorylation of PKC ζ . 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 PKC ζ has not yet been shown. Initially, we found tyrosine phosphorylation of PKC ζ by EGF in several cancer cell lines with high levels of endogenously expressed PKC ζ . Furthermore, to mimic tumorigenic conditions, we used COS-7 cells transiently transfected with EGFR and PKC ζ . In this overexpressing model, we found a time- and concentration-dependent tyrosine phosphorylation of PKC ζ due to activation of EGFR. Tyrosine phosphorylation of PKC ζ essentially involved EGFR tyrosine kinase activity and resulted in catalytic activation of PKC ζ . Furthermore, in the COS-7 cell model as well as in several carcinoma cell lines, we showed the critical involvement of PKC ζ 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 PKC ζ 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, PKC ζ 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 PKC ζ .

To address a putative connection of tyrosine phosphorylation of PKC ζ with the activation of ERK, several experimental strategies have been chosen. First, we generated a novel mutant, PKC ζ -Y417F, which lost three important functional properties compared with the WT PKC ζ . 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 EGFR-mediated stimulation of DNA synthesis. The EGF-induced tyrosine phosphorylation of PKC ζ -Y417F was significantly reduced but not completely abolished, suggesting that in response to EGF, multiple tyrosine residues of PKC ζ are phosphorylated but only tyrosine 417 is critically involved in both the enzymatic activation of PKC ζ and the PKC ζ -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 PKC ζ that are phosphorylated in response to EGF.¹ Furthermore, the catalytically incompetent mutant PKC ζ -T410A, which exhibits the critical tyrosine in position 417, was compared with the novel mutant PKC ζ -Y417F still exhibiting the residue T410, which is critical for the catalytic maturation of PKC ζ 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 PKC ζ , 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 PKC ζ becomes activated by EGF and that both T410 and Y417 are necessary for EGF-induced tyrosine phosphorylation and thereby activation of PKC ζ .

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

¹ H. Daub and C. Liebmann, unpublished results.

four tyrosine residues, 356, 374, 417, and 428, which are highly conserved among the PKC ζ 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 PKC ζ . It should be noted that tyrosine phosphorylation of PKC ζ 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 PKC ζ . For example, the sequence PNPY⁴¹⁷IAP within the structure of PKC ζ might represent a potential consensus sequence for the COOH-terminal SH2 domain of phospholipase C γ 1 (39).

We also compared the EGF-induced activation of ERK in PKC ζ -expressing Mef cells with that in PKC ζ ^{-/-} 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 PKC ζ . Nevertheless, it becomes also evident that the presence of PKC ζ is

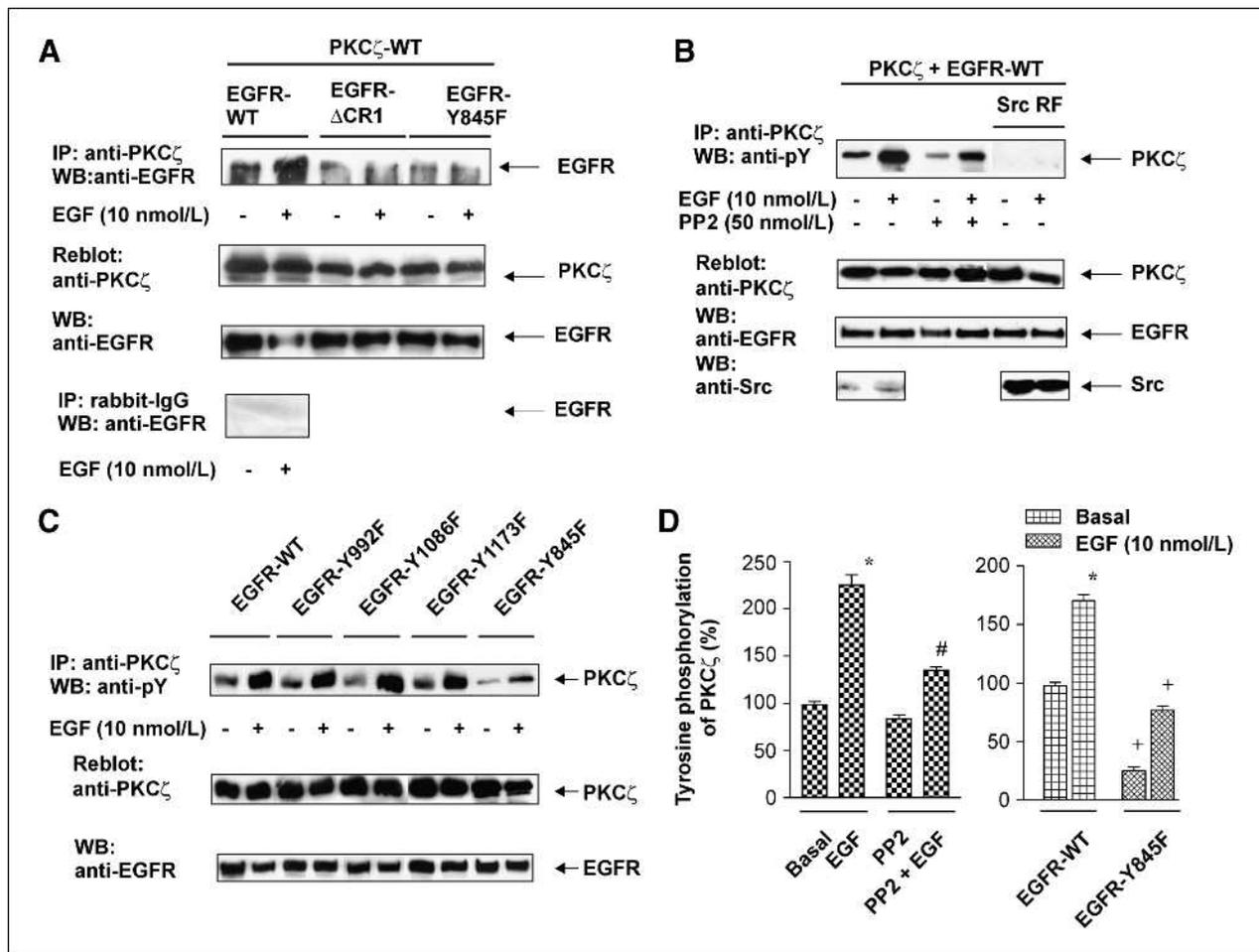


FIGURE 6. Critical role of the Src-specific site pY845-EGFR in mitogenic PKC ζ signaling. A, PKC ζ may physically associate with the activated EGFR. COS-7 cells were cotransfected with PKC ζ and WT EGFR, the dimerization-inactive mutant EGFR- Δ CR1, or the mutant EGFR-Y845F. After serum starvation, the cells were stimulated with 10 nmol/L EGF for 1 minute. Lysates were prepared and immunoprecipitated with anti-PKC ζ 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 PKC ζ 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 PKC ζ 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; PKC ζ 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 PKC $\zeta^{-/-}$ Mef cells sometimes varies between individual experiments. This might explain why in previous studies no differences in ERK activation were found between WT and PKC $\zeta^{-/-}$ cells (33).

In this study, we also investigated how the EGFR might mediate tyrosine phosphorylation and catalytic activation of PKC ζ . Our findings indicate that the EGFR tyrosine kinase is essentially required. Thus, PKC ζ physically associates with the EGFR; the tyrosine phosphorylation is dependent on EGFR dimerization; and the EGFR-specific tyrosine kinase inhibitor AG1478 completely prevents PKC ζ tyrosine phosphorylation. Additionally, the significant inhibition of PKC ζ tyrosine phosphorylation by the Src-inhibitor PP2 and the complete prevention of PKC ζ tyrosine phosphorylation by dominant-negative c-Src suggest the critical involvement of c-Src. Several lines of evidence strongly indicate the dependency of PKC ζ tyrosine phosphorylation on the unique tyrosine residue 845 of EGFR, which is solely phosphorylated through c-Src. Compared with the WT, (a) the coimmunoprecipitation of PKC ζ with the mutant EGFR-Y845F is decreased; (b) the tyrosine phosphorylation of PKC ζ is clearly diminished when the mutant EGFR-Y845F is coexpressed; (c) the catalytic activation of PKC ζ 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 sig-

nificantly more PKC ζ *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 PKC ϵ and postulated a critical role of the C2-domain of PKC ϵ in the recognition of pY845-EGFR (42). Here we show a novel importance of pY845-EGFR as a putative docking site for the aPKC ζ , 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). PKC ζ 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 PKC ζ but corresponds to K355 in

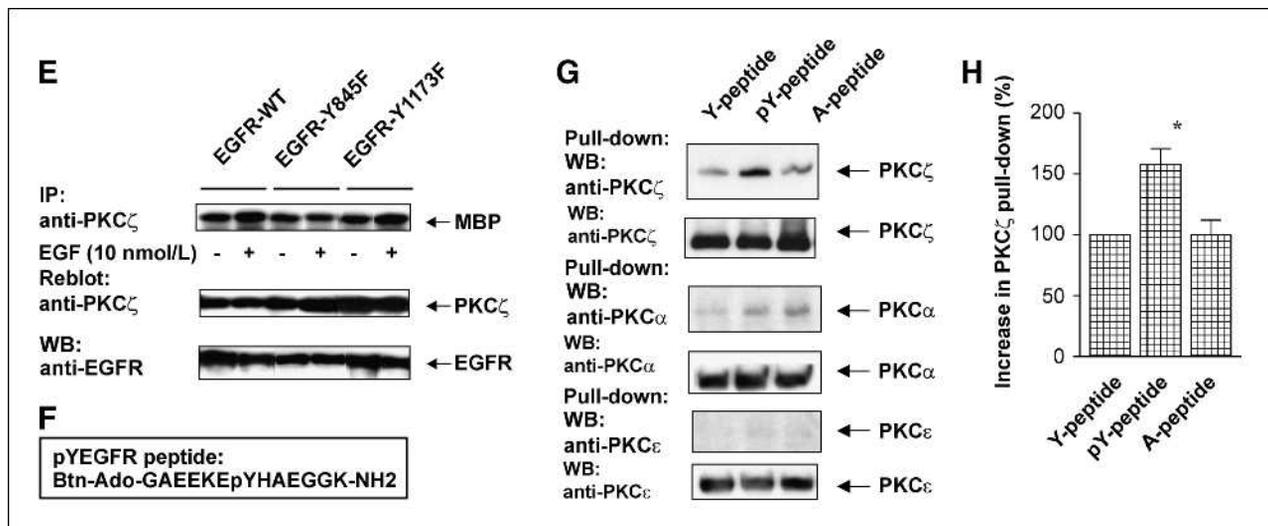


FIGURE 6. Continued. E, COS-7 cells were cotransfected with PKC ζ and WT EGFR, EGFR-Y845F, or EGFR-Y1173F. Serum-starved cells were stimulated with 10 nmol/L EGF for 5 minutes. PKC ζ immunoprecipitates were used for the immune complex kinase assay with MBP as substrate as described in Materials and Methods. MBP phosphorylation was measured by autoradiography and the amount of immunoprecipitated PKC ζ by reblotting with anti-PKC ζ 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 PKC ζ or, for comparison, PKC α or PKC ϵ . 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 μ g 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 PKC ζ pull-down. Columns, means; bars, SD. *, $P < 0.05$, significantly higher. Results from six separate experiments.

p67^{phox}, which plays a critical role in the PB1 domain interaction of p40^{phox}/p67^{phox} (43). It may be speculated that the pY845-dependent association of EGFR and PKC ζ may be mediated by electrostatic interactions between the acidic glutamic acid cluster surrounding Y845 and one of the basic amino acid clusters in PKC ζ , 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 PKC ζ 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 PKC ζ than does the EGFR. Detailed investigations will be necessary to verify this hypothesis.

Taken together, our results suggest that in cells overexpressing PKC ζ , 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 PKC ζ -mediated pathway to ERK. Phosphorylation of the unique tyrosine residue 845 leads to enhanced recruitment of PKC ζ to the EGFR. Then, EGFR-associated PKC ζ becomes tyrosine phosphorylated through EGFR tyrosine kinase and/or c-Src and thereby catalytically activated. Subsequently, PKC ζ may contribute to elevated ERK activation. We pos-

tulate that this pathway occurs cell-specifically and depends on the expression pattern of PKC ζ 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 PKC ζ in several aggressive tumors and highlight the suppression of EGFR-PKC ζ 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- Δ CR1 (Δ 242-259) construct; Dr. K. Pfitzenmaier (Institute of Cell Biology and Immunology, Stuttgart, Germany) for PKC ζ -T410A-EYFP-N1; Dr. M. Leitges (Biotechnology Centre of Oslo, Oslo, Norway) for PKC ζ ^{-/-} Mef cells and Mef WT cells; and Dr. Alexander Berndt (Institute of Pathology, University of Jena, Jena, Germany) for the HNSCC cell line PE/Ca-PJ-15.

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.

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

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