Transactivation of the Epidermal Growth Factor Receptor Is Involved in 12-O-Tetradecanoylphorbol-13-acetate-induced Signal Transduction*

Nanyue Chen‡, Wei-Ya Ma‡, Qing-Bai She‡, Erxi Wu§, Guangming Liu‡, Ann M. Bode‡, and Zigang Dong‡‡

From the ‡Hormel Institute, University of Minnesota, Austin, Minnesota 55912 and the §Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

The mechanism of 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion is still not well understood even though it is thought to be related to the protein kinase C/mitogen-activated protein kinase/AP-1 pathway. Recently, TPA was also found to induce epidermal growth factor receptor (EGFR) activity. Here, we investigated whether the EGFR is a necessary component for TPA-induced signal transduction associated with tumor promotion. We demonstrated that potent inhibitors of the EGFR, PD153035 and AG1478, blocked TPA-induced phosphorylation of extracellular signal-regulated kinases (ERKs), AP-1 activity, and cell transformation. EGFR gene deficiency blocked TPA-induced ERK activity and AP-1 binding activity. The blocking of the ectodomain of the EGFR by a monoclonal antibody depressed TPA-induced ERK activity and AP-1 DNA binding activity. The use of a neutralizing antibody for heparin-binding EGF, one of the ligands of EGFR, blocked TPA-induced phosphorylation of ERKs. BB-94, a potent inhibitor of matrix metalloproteinases, which are activators of ectodomain shedding of EGFR ligands, also blocked TPA-induced ERK activity, AP-1 DNA binding, and cell transformation but had no effect on EGFR-induced signal transduction. Anti-EGFR, anti-heparin-binding EGF, and BB-94 each blocked TPA-induced EGFR phosphorylation, but only anti-EGFR could block EGF-induced EGFR phosphorylation. Based on these results, we conclude that the EGFR is required for mediating TPA-induced signal transduction. EGFR transactivation induced by TPA is a mechanism by which the EGFR mediates TPA-induced tumor promotion-related signal transduction.

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‡To whom correspondence should be addressed: the Hormel Institute, University of Minnesota, 801 16th Ave. NE, Austin, MN 55912. Tel.: 507-457-9640; Fax: 507-457-9606; E-mail: zgdong@hi.umn.edu.

¶The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; MAP, mitogen-activated protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMEM, Eagle’s minimum essential medium; FBS, fetal bovine serum; MMPs, matrix metalloproteinases; ADAMs, proteins with a disintegrin and metalloproteinase domain; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding epidermal growth factor; P, promotion-sensitive; DMEEM, Dulbecco’s modified Eagle’s medium; JNK, Jun

is a highly potent tumor promoter and is used widely for the study of the mechanism of tumor promotion. TPA-induced tumor promotion is characterized by a dramatic increase in cell proliferation (1). This change is primarily the result of promoter-induced alterations in gene expression and in mediation of signal transduction from extracellular stimuli to the nucleus (1). Because protein kinase C (PKC) is an intracellular receptor of TPA, TPA-induced tumor promotion is thought to be related to the PKC/MAP kinases/AP-1 pathway (2–5). However, several reports showed that PKC activation induced by TPA is a transient process, and sustained treatment with TPA down-regulates PKC activity (6–8). On the other hand, TPA-induced tumor promotion is a process of long term exposure. Redig et al. (9, 10) reported recently that PKCa overexpression had no effect on skin tumor promotion induced by TPA, and the overexpression of PKCβ or PKCε reduced the TPA-induced papilloma burden in transgenic mice. These observations suggest that other mechanisms, in addition to the PKC pathway, mediate TPA-induced tumor promotion.

Several investigators recently found that the EGFR was phosphorylated in TPA-promoted skin tumors from SENCAR mice (11) and in TPA-treated cultured cells (12). The pathway mediated by the EGFR is very important in modulating cell proliferation, but no evidence thus far has shown clearly that this pathway is related to TPA-induced tumor promotion. In the present work, we demonstrate that the activation of the EGFR by TPA is a necessary step in TPA-induced signal transduction and cell transformation and therefore is involved in TPA-induced tumor promotion.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimum essential medium (EMEM), gentamicin, and fetal bovine serum (FBS) were from BioWhittaker (Baltimore). l-Glutamine was from Life Technologies, Inc. Luciferase assay substrate was from Promega (Madison, WI). TPA, aprotinin, and leupeptin were from Sigma. EGF, PD153035, and AG1478 were from Calbiochem. BB-94 (an inhibitor of matrix metalloproteinases (MMPs) and ADAMs (proteins with a disintegrin and metalloproteinase domain)) was from British Biotech (Oxford, U. K.).

The antibodies used included rabbit polyclonal phosphorylated ERK antibodies (New England Biolabs, Beverly, MA). rabbit polyclonal EGFR (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal phosphorylated tyrosine (4G10), mouse monoclonal EGFR (neutralizing, clone LA1, Upstate Biotechnologies, Lake Placid, NY), and mouse monoclonal heparin-binding EGF (HB-EGF) (neutralizing, R&D Systems Inc., Minneapolis, MN).

Cell Culture—Mouse epidermal JB6 promotion-sensitive (P⁺) Cl 41 cells were grown at 37 °C in EMEM supplemented with 5% heat-inactivated FBS, 2 mM l-glutamine, and 25 μg/ml gentamicin (13).

N-terminal kinase; BME, basal medium Eagle; TGF-α, transforming growth factor-α.
Mouse epidermal JB6 P⁺ 1-1 cells that were stably transfected with an AP-1 luciferase reporter plasmid were cultured as described previously (13, 14). Human prostate cancer cells, PC3, were grown in 10% FBS and Dulbecco’s modified Eagle’s medium (DMEM) and incubated at 5% CO₂, 37°C.

Primary Embryo Cell Preparation—The mice were sacrificed by cervical dislocation, and uteri filled with embryos were aseptically removed. The embryos were freed from surrounding membranes and minced into little pieces with scissors. One ml of trypsin solution (0.25%) was added, and the embryos were incubated on a dish at 37°C while pipetting the digestion periodically. When the bulk of the suspension consisted of single cells and small clumps of cells, the digestion was stopped by adding 5 ml of DMEM containing 10% FBS. The suspension was transferred to a centrifuge tube, and large chunks were allowed to settle out at room temperature. The supernatant fraction was transferred to a fresh tube and centrifuged at 3,000 rpm for 10 min. The cell pellet was resuspended and distributed into culture flasks at a density of 1–2 x 10⁵ cells/cm². DMEM with 10% FBS was added and the cells incubated at 37°C in a 5% CO₂ atmosphere. The culture medium was changed 8–24 h after the initial plating to remove cellular debris, and

FIG. 1. EGFR inhibitors suppress TPA- or EGF-induced ERK phosphorylation, AP-1 activity, and cell transformation. Panel A, inhibition of TPA- or EGF-induced AP-1 transcriptional activity by EGFR inhibitors. JB6 AP-1 reporter stable P⁺ 1-1 cells (5 x 10⁵/well) were seeded into 96-well plates. After cells reached 80% confluence, they were starved for 24 h by replacing the medium with 0.1% FBS and EMEM. Then the cells were pretreated with PD153035 or AG1478 at various concentrations for 30 min and treated with 20 ng/ml TPA or EGF for another 24 h. AP-1 activity was measured with a luciferase activity assay as described under “Experimental Procedures.” The results are presented as a percent induction compared with control, and data are shown as the means ± S.E. of four wells. Panel B, effect of EGFR inhibitors on TPA- or EGF-induced ERK phosphorylation. JB6 Cl 41 cells (8 x 10⁴/well) were cultured in a monolayer in six-well plates until they reached 90% confluence, and then the cells were starved for 48 h in 0.1% FBS and EMEM. Cells were pretreated with PD153035 or AG1478 at various concentrations, as indicated, for 30 min and then treated with 20 ng/ml TPA or 20 ng/ml EGF for 15 min and harvested with SDS sample buffer. The samples were analyzed by Western blotting with antibodies against nonphosphorylated or phosphorylated ERKs (New England Biolabs). Panel C, inhibition of cell transformation by EGFR inhibitors. Cl 41 cells (1 x 10⁴ cells) were or were not exposed to 20 ng/ml TPA or 20 ng/ml EGF and different concentrations of PD153035 or AG1478 in 1 ml of 0.33% BME agar containing 10% FBS over 3 ml of 0.5% BME agar containing 10% FBS in each well of six-well plates. The cultures were maintained in a 37°C, 5% CO₂ incubator for 4 weeks. Cell colonies were scored by microscope with a computer analysis system. The results are presented as a percent of control colony count. Data are shown as the means ± S.E. of triplicate wells.
confluence. The cells were then starved for 24 h in FBS-free DMEM.

The upper band (450 base pairs) corresponds to the mutant allele.

Polymerase chain reaction. DNA was isolated from the embryonic cells.

/analysis of genomic DNA from wild-type and homozygous (H11002/H11002).

Panel A

Phosphorylation of ERKs and AP-1 DNA binding activity.

EgfrWT cells were cultured in a monolayer in six-well plates until they reached 90% confluence. Then the cells were starved for 24 h in FBS-free DMEM. The cells were treated with inhibitors for 30 min and cultured with TPA or EGF for 24 h and harvested by adding lysis buffer (100 mM K2HPO4, pH 7.8, 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA). Luciferase activity was measured by a luminometer (Monolight 2010, Analytical Luminescence Laboratory), and relative AP-1 activity was calculated as described previously (13).

Nuclear Protein Analysis—Gel shift assays were used to detect AP-1 DNA binding activity. Nuclear extracts were prepared as described previously (4). In brief, the cells were disrupted with 500 μl of lysis buffer (50 mM KCl, 0.5 mM Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 μg/ml aprotinin). After centrifugation at 14,000 rpm in a micro-centrifuge for 1 min, the nuclei were washed with 500 μl of the same buffer but without Nonidet P-40, then placed into 200 μl of extraction buffer (the same concentration of the other reagents as in the lysis buffer except 500 mM KCl and 10% glycerol). After centrifugation at 14,000 rpm for 5 min, the supernatant fraction was stored at 70 °C. An AP-1 binding sequence from the human collagenase promoter region, 5’-AGCATGAGTCAGA-

CACCTCTGGGC-3’, was synthesized and labeled with [32P]dCTP using the Klenow fragment (Life Technologies, Inc.). Nuclear protein (3 μg) was added to the DNA-binding buffer, which contained 5 × 10^4 cpm [32P]-labeled oligonucleotide probe, 1.5 μg of poly(dI-dC), and 3 μg of bovine serum albumin. The reaction mixture was incubated on ice for 10 min followed by incubation at room temperature for 20 min. The DNA-protein complexes were resolved in a 6% nondenaturing acrylamide gel. The gel was dried and scanned using the Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Western Blotting—Samples containing equal amounts of protein were resolved on an 8% SDS-polyacrylamide gel, and proteins were subsequently transferred and analyzed as described previously (16). Immunoblot analysis of ERK and JNKs proteins was carried out using MAP kinase antibodies against ERKs and JNKs as described previously (16). Antibody-bound proteins were detected by chemiluminescence (ECF of Amersham Pharmacia Biotech) and analyzed using the Storm 840 PhosphorImager.

Anchorage-independent Transformation Assay—JB6 Cl 41 cells (1 × 10^6) were exposed to TPA or EGF, with or without different concentrations of inhibitors, in 1 ml of 0.33% BME agar containing 10% FBS over 3 ml of 0.5% BME agar containing 10% FBS in each well of a six-well plate. The cultures were maintained in a 37 °C, 5% CO2 incubator for 4 weeks, and then the cell colonies were scored automatically by microscope with a computer analysis system.

Immunoprecipitation of EGFR—PC3 cells (10^6) were seeded in 150-mm dishes. Cells grown to 80–90% confluence were starved in FBS-free DMEM for 36 h and treated with inhibitors or agonists as indicated. Cells were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na3VO4, 1 mM NaF) for 15 min at 4 °C with slow rotation. Equal amounts of protein were immunoprecipitated with a rabbit polyclonal antibody for EGFR and protein A/G beads. The precipitates were washed and suspended in SDS sample buffer. Samples were resolved on 6% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against phosphorylated tyrosine (4G10) or EGFR. Antibody-bound proteins were detected by chemiluminescence (ECF) and analyzed using the Storm 840 PhosphorImager.

FIG. 2. Deficiency of the Egfr gene blocks TPA-induced phosphorylation of ERKs and AP-1 DNA binding activity. Panel A, analysis of genomic DNA from wild-type and homozygous (+/-) mice by polymerase chain reaction. DNA was isolated from the embryonic cells. The upper band (450 base pairs) corresponds to the mutant allele (Egfr-/-), and the lower band (350 base pairs) corresponds to the wild-type allele. Panel B, Western blot analysis of embryonic cell lysates. The blots were probed with an antibody to EGFR. Panel C, EGFR deficiency blocks TPA-induced EGFR phosphorylation. EGFR wild type (WT) and EGFR deficient (Egfr-/-) cells (8 × 10^4/well) were cultured in a monolayer in six-well plates until they reached 90% confluence. Then the cells were starved for 24 h in FBS-free DMEM. The cells were then cultured with TPA or EGF for 24 h and harvested by adding lysis buffer (100 mM K2HPO4, pH 7.8, 1% Triton X-100, 1 mM dithiothreitol, and 2 mM EDTA). Luciferase activity was measured with a luminometer (Monolight 2010, Analytical Luminescence Laboratory), and relative AP-1 activity was calculated as described previously (13).

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EGFR Inhibitors Depress TPA-induced ERK Phosphorylation, AP-1 Activity, and Cell Transformation—Although the tumor promotion activity of TPA is considered to be mediated mainly by PKC, activation of the EGFR tyrosine kinase has been found to increase in mouse epidermis with multiple topical treatments of TPA (11). Also, the expression of EGFR ligands such as transforming growth factor-α (TGF-α) and HB-EGF is enhanced in TPA-treated mouse skin (17). However, whether EGFR is involved in TPA-induced tumor promotion or cell transformation is not clear. Here, we used PD153035 and AG1478, potent and specific inhibitors of tyrosine phosphorylation of the EGFR, to investigate whether the EGFR is involved in TPA-induced signal transduction and cell transformation. We found that either PD153035 or AG1478 blocked TPA-induced AP-1 activity, but the effect on TPA was weaker than that of EGF (Fig. 1A). In agreement with our previous reports, TPA or EGF induces AP-1 transcriptional activity in JB6 cells by 2.9-fold and 2.0-fold, respectively (5, 14). At a 500 nM concentration of either inhibitor, EGF-induced AP-1 activation was totally blocked, whereas TPA-induced AP-1 activity was blocked by 70% (p < 0.05). This suggests that TPA-induced AP-1 activity is also partially mediated by the EGFR.

MAP kinases are upstream activators of AP-1 transcriptional activation. To determine by which pathway the EGFR mediates TPA-induced AP-1 activity, we studied the effect of specific EGFR inhibitors on TPA-induced MAP kinase activity. Previous studies have shown that of the three MAP kinases, ERKs are the main phosphorylation target of TPA. The results in Fig. 1B show that PD153035 and AG1478 can completely block both EGF- and TPA-induced phosphorylation of ERKs.

TPA and EGF both induce JB6 cell transformation. In general, the effect is thought to be related to activation of transcription factor AP-1 because the transformation only occurs in the P+ cells in which TPA strongly induces AP-1 activity (2, 18). Blocking AP-1 activation by a dominant negative c-Jun also blocks TPA-induced cell transformation (2). To address whether the EGFR is involved in TPA-induced cell transformation, we tested the effect of EGFR inhibitors on TPA-induced cell transformation. We found that EGF-induced cell transformation was inhibited 90–100% by either PD153035 or AG1478. Both inhibitors also blocked TPA-induced cell transformation, but only by 50–60% (Fig. 1C). Taken together, these results suggested that the EGFR is required for partially mediating TPA-induced AP-1 signal transduction and cell transformation.

Deficiency of the EGFR Gene Blocks TPA-induced Phosphorylation of ERKs and AP-1 DNA Binding Activity—To investigate further the role of the EGFR in mediating TPA-induced signal transduction, we used EGFR knockout (Egfr−/−) embryo cells to compare with wild-type EGFR cells (Egfr+/+). EGFR heterogenous mice (Egfr+/−) were from Jackson Laboratory (Bar Harbor, ME). The genomic DNA phenotype and protein expression of the EGFR in knockout cells were confirmed by polymerase chain reaction and Western blotting, respectively. The results shown in Fig. 2A indicate that a DNA fragment of 450 base pairs (−/−) replaced the normal fragment of 350 base pairs (+/+), agreeing with results of others. Egfr−/− cells did not express the EGFR protein (Fig. 2B). These results indicated that the knockout of the Egfr gene was effective.

We tested the effect of EGFR deficiency on TPA-induced MAP kinase activity and AP-1 DNA binding activity using Egfr−/− cells. The results in Fig. 2C show that in Egfr−/− cells, TPA-induced phosphorylation of ERKs was inhibited mark-
reached 90% confluence, and then they were starved for 24 h in 0.1% nonphosphorylated or phosphorylated ERKs. Samples were analyzed by Western blotting with antibodies against ng/ml EGF for 10 min and harvested with SDS sample buffer. The concentrations for 30 min and then treated with 20 ng/ml TPA or 20 ng/ml EGF for 10 min and harvested with SDS sample buffer. The concentrations for 30 min and then treated with 20 ng/ml TPA or 20 ng/ml EGF for 10 min and harvested with SDS sample buffer. The concentrations for 30 min and then treated with 20 ng/ml TPA or 20

A Neutralizing Antibody for EGFR Blocked TPA-induced ERK Phosphorylation and AP-1 DNA Binding Activity—The protein structure of the EGFR consists of three segments, including an extracellular region that functions as a binding site for ligands, a transmembrane region, and an intracellular region that is a tyrosine kinase (receptor tyrosine kinase). The main effect of EGFR inhibitors is to repress the receptor tyrosine kinase activity of the EGFR. Because many stimuli may activate receptor tyrosine kinase directly, the results showing that inhibitors decrease TPA-induced signal transduction do not exclude the possibility that TPA activates other targets that lead to direct activation of receptor tyrosine kinase. To determine how the EGFR mediates TPA-induced signal transduction, we designed an experiment to block the binding of ligands to the extracellular region of the EGFR. A neutralizing antibody for the EGFR binds to the extracellular domain of the EGFR and competes for binding of ligands to the EGFR on cells, which blocks the biological effects elicited by ligands but does not activate the tyrosine kinase activity of the EGFR (19). Because this monoclonal antibody only reacts with human EGFR, we used the PC3 human prostate cancer cell line. The results in Fig. 3A indicate that the neutralizing antibody blocked EGF-induced EGFR phosphorylation, suggesting that this antibody is effective. Pretreating cells with this antibody also inhibited TPA-induced EGFR phosphorylation (Fig. 3A), ERK phosphorylation, and AP-1 DNA binding (Fig. 3, B and C). These results provide strong evidence that TPA-induced signal transduction is related to the binding of certain ligands to the EGFR.

A Neutralizing Antibody for HB-EGF Blocked TPA-induced Phosphorylation of ERKs—Six EGF-like mammalian gene products are known to activate the EGFR directly and include EGF, TGF-α, HB-EGF, amphiregulin, betacellulin, and epiregulin (20). These molecules are expressed as transmembrane protein precursors and are subject to proteolytic cleavage of their extracellular domains to produce soluble growth factors. The processing, also referred to as “ectodomain shedding,” of these precursor molecules can activate the EGFR. TPA was previously demonstrated to induce the processing of a number of membrane-anchored proteins including TGF-α (21), HB-EGF (22, 23), and amphiregulin (24). This suggests that TPA may activate the EGFR through the ectodomain shedding of these precursors. To demonstrate further that the release of EGFR ligands is involved

Fig. 5. BB-94 suppresses TPA- but not EGF-induced ERK phosphorylation, AP-1 DNA binding, and cell transformation. Panel A, effect of BB-94 on TPA- or EGF-induced ERK phosphorylation. JB6 Cl 41 cells (8 × 10⁴/well) were cultured in a monolayer in six-well plates until they reached 90% confluence. Then they were starved for 48 h in 0.1% FBS and EMEM. The cells were or were not exposed to 20 ng/ml TPA or 20 ng/ml EGF for 10 min and harvested with SDS sample buffer. The samples were analyzed by Western blotting with antibodies against nonphosphorylated or phosphorylated ERKs. Panel B, effect of BB-94 on TPA- or EGF-induced AP-1 DNA binding activity. JB6 Cl 41 cells were cultured in a monolayer in 10-cm dishes (1 × 10⁶/dish) until they reached 90% confluence, and then they were starved for 24 h in 0.1% FBS and DMEM. The cells were or were not exposed to 20 ng/ml TPA or 20 ng/ml EGF for 10 min and harvested with SDS sample buffer. The samples were analyzed by Western blotting with antibodies against nonphosphorylated or phosphorylated ERKs. Panel C, effect of BB-94 on TPA- or EGF-induced AP-1 DNA binding activity. JB6 Cl 41 cells (1 × 10⁵ cells) were or were not exposed to 20 ng/ml TPA or 20 ng/ml EGF with 5 μM or 10 μM BB-94 or 1 μM PD153035 in 1 ml of 0.33% BME agar containing 10% FBS in each well of six-well plates. The cultures were maintained in a 37°C, 5% CO₂ incubator for 4 weeks. Cell colonies were scored by microscope with a computer analysis system. The results are presented as a percent of control colony count, and data are shown as the means ± S.E. of triplicate wells. Panel D, effect of BB-94 on EGF- or TPA-induced EGFR phosphorylation. PC3 cells were cultured in a monolayer in 150-mm dishes until they reached 90% confluence and then starved in serum-free DMEM for 36 h. The cells were or were not exposed to 5 μM BB-94 for 30 min and then treated with 600 ng/ml TPA or 10 ng/ml EGF for 3 min. The samples were immunoprecipitated by using anti-EGFR and probed with anti-phosphotyrosine (4G10) or anti-EGFR. 
DISCUSSION

The strong tumor-promoting effect of TPA is believed to contribute to its activation of PKC. However, TPA has also been shown to increase phosphorylation of the EGFR (11, 12). These data suggest that TPA-stimulated signal transduction may be related to a signaling network that couples TPA to the EGFR. However, the role of the EGFR in TPA-induced signal transduction is not clear. Here we demonstrated that repression of the EGFR by specific inhibitors reduced TPA-induced phosphorylation of ERKs, AP-1 activity, and cell transformation. EGFr-deficient cells blocked TPA-induced ERK activity and AP-1 DNA binding activity. Blocking of the ectodomain of the EGFR by a monoclonal antibody hindered TPA-induced EGFR phosphorylation, ERK activity, and AP-1 DNA binding activity, suggesting that TPA induced the release of some components functioning as EGFR ligands. In addition, the use of an antibody to neutralize HB-EGF, a kind of EGFR ligand, suppressed TPA-induced EGFR phosphorylation and ERK activity. Inhibition of MMP, the activator of ectodomain shedding of EGFR ligands, blocked TPA-induced EGFR phosphorylation as well as ERK activity and cell transformation. Taken together, these results suggest that the EGFR is involved in mediating TPA-induced signal transduction. Induction of EGFR transactivation by TPA is a mechanism by which the EGFR mediates TPA-induced tumor promotion-related signal transduction.

PKC is a major target of TPA and, thus, the role of PKC in mediating tumor promotion has been emphasized. Activated PKC phosphorylates target proteins on serine or threonine residues, presumably altering their functional properties. MAP kinases are important mediators of signal transduction from various extracellular stimuli to many cellular processes such as proliferation and differentiation. Activation of PKC by TPA results in the rapid activation of ERKs (26–28). ERKs induce c-Fos and c-Jun expression, resulting in increased transcriptional activity of AP-1 (29–31). AP-1 is an important nuclear transcription factor consisting of c-Jun and c-Fos proteins (32, 33). The activation of signal transduction pathways that lead to stimulation of AP-1 activity seems to be a common mechanism for diverse types of tumor promoters (2, 34, 35). Blocking AP-1 activity by retinoids and by expression of a transactivation domain deletion mutant of c-Jun, TAM67, prevents TPA-induced cell transformation in JB6 cells and tumor promotion in a mouse skin model (2–4, 36).

Evidence is emerging that suggests an important role for signaling through the EGFR in multistage carcinogenesis (37). Topical application of TPA on SENCAR mice resulted in a significant increase in binding of 125I-EGF to its receptor (37). Moreover, TPA has been found to up-regulate protein and mRNA expression of the EGFR and its ligands, TGF-α and HB-EGF, in both cultured keratinocytes and mice with skin tumors (17, 37). In the present work, we demonstrated that knockout of the Egfr gene or inhibition of EGFR activation by EGFR inhibitors blocked TPA-induced ERK phosphorylation, AP-1 activity, and cell transformation. Even though blocking EGFR did not completely inhibit TPA-induced signal transduction, the EGFR appears to be required for mediating TPA-induced tumor promotion-associated signal transduction.

The mechanism of TPA-induced EGFR activation is not well understood. Some studies have shown that EGFR was involved in the signaling networks activated by stimuli that do not interact directly with this receptor (38). These stimuli include G protein-coupled receptor agonists thrombin and lysophosphatidic acid (39), calcium (40), and UV irradiation (41). The process by which EGFR-unrelated stimuli may release some EGFR ligands leading to EGFR activation is termed EGFR transactivation (38).

Recent studies on the transactivation of the EGFR by G protein-coupled receptor agonists have shown that the stimulation of a protease activity by these agonists is involved in the
transactivation (42). This stimulation results in the cleavage of EGF-like precursors and the production of diffusible growth factors that then activate the EGFR (12, 38, 39). This proteolytic event is likely to be mediated by a metalloproteinase, based on sensitivity of the transactivation of EGFR to BB-94, a broad spectrum metalloproteinase inhibitor (42). TPA has been suggested to activate cleavage of pro-TGF-α, pro-HB-EGF, and proamphiregulin (22–24). The cleavage of pro-HB-EGF was also found to be modulated by metalloproteinase-disintegrins (ADAM family), members of the Metzincin family (23, 43). In the present work, we used a neutralizing antibody against HB-EGF or EGFR and suppression. Through blocking ligand binding to the EGFR by using the EGFR for its ligands, resulting in inhibition of TPA-induced transactivation (42). This stimulation results in the cleavage of EGFR and ERK phosphorylation. Moreover, BB-94, a potent inhibitor of MMPs and ADAMS, blocked TPA-induced ERK activity and cell transformation but did not affect EGF-induced signal transduction. These results suggest that TPA-induced ectodomain shedding of certain EGFR ligands by MMP activates the EGFR, and this transactivation of the EGFR is an important mechanism by which the EGFR mediates TPA-induced tumor promotion-associated signal transduction.

To address whether the EGFR is involved in TPA-induced signal transduction and cell transformation, we used EGFR-deficient cells and highly specific EGFR inhibitors to provide direct evidence that the EGFR, at least partially, is required for TPA-induced signal transduction associated with cell transformation. Through blocking ligand binding to the EGFR by using a neutralizing antibody against HB-EGF or EGFR and suppressing MMP activity by using a MMP inhibitor, we demonstrated that TPA-induced transactivation of the EGFR occurs through the release of EGFR ligands. Taken together, our data suggest that EGFR mediates TPA-induced tumor promotion-associated signal transduction (Fig. 6). This mechanism may account for TPA-induced long-term tumor promotion and thus provide a novel mechanism of TPA-induced tumor promotion.

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Nanyue Chen, Wei-Ya Ma, Qing-Bai She, Erxi Wu, Guangming Liu, Ann M. Bode and Zigang Dong

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