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Theaflavin-3, 3'-Digallate Induces Epidermal Growth Factor Receptor Down-Regulation

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Abstract

Black tea is one of the most popular beverages worldwide and especially in Western nations. Theaflavins, a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3, 3'-digallate (TF-3) are the major components of black tea. Among these black tea components, theaflavin is generally considered to be the more effective component for the inhibition of carcinogenesis. Recently, TF-3 has been shown to have an antiproliferative effect on tumor cells, but the mechanism is not clear. In this study, we showed that TF-3 induced internalization and down-regulation of the epidermal growth factor receptor (EGFR). These results suggested that TF-3 induces EGFR endocytosis and degradation. We further showed that TF-3 stimulated EGFR ubiquitination and tyrosine kinase activation. Interestingly, TF-3-induced EGFR down-regulation is inhibited by the proteasome inhibitor, MG132, but not by the EGFR specific receptor tyrosine kinase inhibitor, AG1478. Furthermore, pretreatment with TF-3 inhibited EGF-induced EGFR autophosphorylation, ERKs phosphorylation and AP-1 activation in JB6 Cl41 cells. In addition, TF-3 inhibited EGF-induced anchorage-independent cell transformation. Overall, our results indicate that TF-3 might exert chemopreventive effects through the down-regulation of the EGFR.

Keywords

theaflavins; JB6 Cl41 mouse epidermal skin cells; A431 human epidermoid carcinoma; anchorage-independent cell transformation

Abbreviations

TF-3, mixture of theaflavin, theaflavin-3-gallate, theaflavin-3'-gallate and theaflavin-3, 3'-digallate; EGFR, epidermal growth factor receptor; TPA, 12-O-tetradecanoylphorbol-13-acetate; AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; BME, Basal medium Eagle; FBS, fetal bovine serum; HEK, human embryonic kidney

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INTRODUCTION

Tea (*Camellia sinensis*) is one of the most popular beverages worldwide. Many studies have shown that green tea, black tea, and tea polyphenol preparations inhibit carcinogenesis [1-4]. Among the black tea components, theaflavins, a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3'-gallate (TF-2b), and theaflavin-3,3'-digallate (TF-3), are generally considered to be the effective components for inhibition of cancer development [1, 2]. Liang *et al.* [5] showed that TF-3 inhibits the proliferation of certain tumor cells. We previously showed that theaflavins inhibit 12-*O*-tetradecanylphorbol-13-acetate (TPA), epidermal growth factor (EGF) or UVB-induced activator protein-1 (AP-1) activation [6,7] and TPA- or EGF-induced anchorage-independent cell transformation [6]. In addition, Halder and Bhaduri [8] reported that theaflavins have antioxidative activity in human blood cells. These reports demonstrated that theaflavins are useful for cancer chemoprevention. However, further study is still needed to clarify the molecular mechanisms and targets of the chemopreventive effects of theaflavins.

The EGF receptor (EGFR), one of the receptor tyrosine kinases, plays a pivotal role in regulating cell proliferation, differentiation, and transformation [9-11]. The EGFR is an important target for cancer therapy [12]. Many carcinomas are promoted by EGFR activation, which can result from mutation of the receptor [13,14], its overexpression [15-17], or from EGFR stimulation through autocrine loops [18].

In this study, we found that TF-3, an important component of theaflavins, induces EGFR down-regulation. We hypothesized that the down-regulation of the EGFR may be an important mechanism of the anticancer activity of TF-3 and that TF-3 may induce receptor down-regulation through the ubiquitination and degradation of the EGFR. Our results confirmed that TF-3 inhibits EGFR signaling and EGF-induced anchorage-independent cell transformation, findings that should contribute to a clearer understanding of cancer chemoprevention by theaflavins.

MATERIALS AND METHODS

Chemicals

The black tea polyphenols, a mixture of TF-1, TF-2a, TF-2b and TF-3 (provided by Thomas J. Lipton Co., Englewood Cliffs, NJ), were separated by chromatography on a LH-20 column to a purity of >98%. The structures of these tea polyphenols are shown in Figure 1A. TF-1, TF-2a, TF-2b or TF-3 were dissolved in ethanol and the final concentration of ethanol in the culture medium was < 0.1%.

Reagents

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), penicillin, streptomycin, L-glutamine and Protein A-agarose were obtained from Invitrogen (Carlsbad, CA). Basal medium Eagle (BME), EGF and the β -actin antibody were obtained from Sigma (St. Louis, MO). AG1478, a specific EGFR tyrosine kinase inhibitor [19], and MG132 (Z-Leu-Leu-Leu-CHO), a proteasome inhibitor [20], were from Calbiochem-Novabiochem Corp. (San Diego, CA). Fetal bovine serum (FBS) was from Gemini Bio-Product (Calabasas, CA). The antibody against ubiquitin was from Upstate (Charlottesville, VA) and antibodies against EGFR (528), EGFR (1005) and phospho-tyrosine (p-Tyr) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against p44/p42 MAPK (ERK) and phospho-ERKs were from Cell Signaling Technology Inc. (Beverly, MA). Anti-mouse IgG conjugated with Alexa Fluor 568 and biotinylated EGF were from Molecular Probes Inc. (Eugene, OR). The IRDye™ 800-conjugated secondary antibody was from Rockland Inc.

(Gilbertsville, PA). The DAPI/antifade solution was from Chemicon International Inc. (Temecula, CA).

Cell Culture

Human A431 epidermoid carcinoma cells and the human embryonic kidney (HEK) epithelial cell line 293 were cultured in DMEM supplemented with 10% heat-inactivated FBS and glutamine (2 mM) at 37 °C in a humidified atmosphere of 5% CO₂. Mouse epidermal JB6 Cl41 cells were cultured in MEM containing 5% FBS and 2 mM glutamine.

Plasmids and Transfection

Wild type EGFR [10] and the Y1045F mutant of EGFR [21] in pcDNA3 were gifts from Yosef Yarden (The Weizmann Institute of Science, Rehovot, Israel). Transfection into HEK 293 cells was performed using the SuperFect transfection reagent (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions.

Immunofluorescence

After culturing for 24 h, media were replaced with serum-free DMEM and A431 cells were incubated for 24 h. A431 cells were incubated with TF-3 (20 µM) or 0.05% ethanol, as a control, for 1 h. Cells were then washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min, washed with PBS twice, permeabilized with 0.5% Triton X-100 for 10 min, and washed with 0.02% Tween 20 in PBS twice. After being blocked for 30 min in 2% BSA in PBS, the cells were incubated with the EGFR (528) antibody (1:100 dilution in blocking buffer) at 37 °C for 45 min, and then washed with 0.02% Tween 20 in PBS twice and incubated with anti-mouse IgG conjugated with Alexa Fluor 568 (1:500 dilution in blocking buffer) at 37 °C for 45 min. The cells were washed and mounted with DAPI/antifade solution in PBS. Images were taken with a confocal microscope, Olympus Fluoview FV500 Laser Scanning confocal system (Olympus, Tokyo, Japan).

Cell Lysate Preparation and Immunoprecipitation

Cells were cultured to 80% confluence and then starved in serum-free DMEM for 24 h in an incubator at 37 °C and 5% CO₂, and the cells were treated with TF-3 for various times or concentrations. After washing with PBS, cells were harvested and disrupted with cell lysis buffer [20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA), 1 mM ethylene glycol-bis (2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The lysed samples were transferred to microcentrifuge tubes and sonicated for 5 seconds 4 times on ice, and then cleared by centrifugation (13,000 rpm, 15 min) at 4 °C. Protein amounts in lysates were measured by the D_C protein assay kit (Bio-Rad, Hercules, CA). For immunoprecipitation, cell lysates adjusted to 1mg/ml protein were precleared by protein A-agarose beads. After gentle rocking at 4 °C for 1 h, beads were removed by centrifugation. The EGFR (528) antibody, which recognizes the N-terminus of the EGFR, was added to the lysates and then incubated with gentle agitation at 4 °C for 2 h. Then, protein A-agarose beads were added to the lysate/antibody mixture, and incubated with gentle agitation at 4 °C, overnight. The immunoprecipitates were collected by centrifugation at 4 °C and washed 4 times with cell lysis buffer, then boiled for 5 min with 50 µl of 2 × SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue).

Immunoblotting

For direct electrophoretic analysis, 3 × SDS sample buffer was added to cell lysates. The samples were loaded on SDS-polyacrylamide gels for electrophoresis and subsequently

transferred onto polyvinylidene fluoride membranes (Millipore; Bedford, MA). After blocking, the membranes were incubated with the specific primary antibody with gentle agitation overnight at 4 °C. After washing, the membranes were incubated with IRDye™ 800-conjugated secondary antibody for 1 h at room temperature. Detection was performed using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE)

Receptor Down-Regulation Assay

A431 cells were seeded in 96-well plates (3×10^4 cells). After culturing for 24 h, media were replaced with serum-free DMEM and cells were incubated for 24 h. A431 cells were incubated with TF-3 at various concentrations or 0.05% ethanol, as a control, for 1 h. After washing with PBS, cells were incubated at 4 °C with 150 mM acetic acid (pH 2.7), containing 150 mM NaCl to remove receptor bound ligand [22]. The number of ligand binding sites on the cell surface was determined by incubating cells with biotinylated EGF [23]. To quantify the amount of biotinylated EGF, cells were incubated with streptavidin-horseradish peroxidase (HRP) (Zymed Laboratories, Carlsbad, CA) followed by HRP substrate (Bio-Rad) to develop color. The reaction was stopped by adding 2% oxalic acid and measuring the absorbance (OD₄₁₅) by microplate photometer (Multiskan EX). The blank (no incubation with biotinylated EGF) was subtracted from each respective absorbance reading. The percentage of cell surface receptors was calculated using the following formula: (% of cell surface receptors) = $\text{OD}_{415}(\text{TF-3 treated cells})/\text{OD}_{415}(\text{control cells}) \times 100$.

Assay for AP-1 Activity

JB6 AP-1-luciferase promoter stably transfected cells (P⁺1-1) were used for assay of AP-1 activity. Viable cells (8,000 cells) suspended in 100 µl of 5% FBS/MEM were added to each well of a 96-well plate. After incubation for 24 h, cells were starved in 0.1% FBS/MEM for 24 h. The cells were treated with TF-3 at various concentrations or 0.05% ethanol, as a control, for 1 h and subsequently exposed to EGF (10 ng/ml) and incubated for an additional 24 h. The cells were disrupted, and luciferase activity was measured by using a luminometer (Monolight 2010) as described previously [24,25].

Anchorage-Independent Transformation Assay

JB6 Cl41 cells (8,000 cells) were exposed to EGF (10 ng/ml) with or without different concentrations of TF-3 in 1 ml of 0.33% BME agar over 3 ml of 0.5% BME agar containing 10% FBS. The cultures were maintained in an incubator at 37 °C and 5% CO₂ for 14 days, and the cell colonies were scored using a microscope and the Image-Pro PLUS computer software program (Media Cybernetics; Silver Spring, MD) as described by Colburn *et al.* [26,27].

Statistics

Significant differences between groups were determined by one-way ANOVA and pairwise comparisons were conducted using Fisher's PLSD test.

RESULTS

Theaflavins Induce EGFR Down-Regulation

We previously showed that theaflavins inhibited EGF-induced AP-1 activation and malignant transformation in mouse epidermal JB6 Cl41 cells [6]. In the current study, we investigated the effects of individual theaflavins (Figure 1A) on the EGFR in mouse skin epidermal JB6 Cl41 cells and A431 cells, an EGFR overexpressing human epidermoid carcinoma cell line. Immunoblotting was performed with anti-EGFR (1005), which recognizes the C-terminus of the EGFR. These two cell lines provide a convenient model to compare human skin and mouse cells, which express varying levels of the EGFR. Results indicated that treatment with

theaflavins decreased the EGFR total protein level (Figure 1B), suggesting that the C-terminus of the EGFR was degraded by exposure to some of the theaflavins. In particular, TF-3 dramatically decreased the level of the EGFR in both cell lines.

To delineate the localization of the EGFR after TF-3 treatment, we performed immunofluorescence analysis by confocal microscope using anti-EGFR (528), which recognizes the extracellular (the N-terminus) of the EGFR, which is not affected by the proteasome [21]. In control cells, the EGFR was localized in the plasma membrane (Figure 2A, left panel). On the other hand, after treatment with TF-3 for 1 h, the EGFR was found to be not only in the membrane, but also in the cytosol (Figure 2A, right panel). We further examined the number of EGFRs on the cell surface after treatment with TF-3. EGFR relative number was determined by the binding level of biotinylated EGF. As shown (Figure 2B), treatment with TF-3 (20 μ M) significantly decreased EGFR number on the cell surface. These results suggested that TF-3 induces internalization and endocytosis of the EGFR.

TF-3 Induces Ubiquitination and Proteasome Degradation of the EGFR

EGF binding to the EGFR is known to induce EGFR down-regulation [28] by inducing endocytosis and subsequent receptor degradation or recycling [29]. Once tagged by ubiquitin, the EGFR (C-terminus) is destined to intracellular degradation that can be partially inhibited by proteasome inhibitors [10]. The ubiquitin/proteasome pathway is reported to be involved in EGF-induced EGFR degradation [11,30]. We therefore examined whether EGFR down-regulation was accompanied by receptor ubiquitination. Results indicate that TF-3 induced down-regulation and ubiquitination of the EGFR in a time-dependent manner in A431 cells (Figure 3A). Then, we determined whether inhibition of the proteasome would impair TF-3-induced EGFR down-regulation. Cells were first incubated with or without a proteasome inhibitor, MG132, and then exposed to TF-3 (Figure 3B). Preincubation with MG132 (10 μ M) for even 1 or 6 h inhibited TF-3-induced EGFR down-regulation. Furthermore, we performed the same experiment in the presence of cycloheximide (CHX, 10 μ g/ml) to determine whether TF-3-induced EGFR down-regulation was related to an inhibition of protein synthesis. Cycloheximide had no effect on EGFR down-regulation induced by TF-3 or on the inhibitory effect of MG132, indicating that TF-3-induced EGFR down-regulation is not the result of inhibition of protein synthesis. These results suggest that TF-3-induced ubiquitination of the EGFR leads to receptor down-regulation through receptor degradation by the proteasome.

TF-3-Induced EGFR Down-Regulation Does Not Require Receptor Tyrosine Kinase Activation

Liang *et al.* [5] showed that TF-3 competes with EGF for binding to the EGFR in A431 cells. Therefore, we examined whether TF-3 induces EGFR intrinsic tyrosine kinase activation. TF-3 induced EGFR tyrosine phosphorylation, although not as markedly as phosphorylation induced by EGF (Figure 4A). Phosphorylation levels were increased at 15 min and then decreased. This result suggested that TF-3 may bind to the EGFR acting as an agonist. Tyrosine kinase activation is critical for EGFR down-regulation by EGF binding to the receptor [29]. In the presence of AG1478, a specific inhibitor of EGFR intrinsic tyrosine kinase activity, phosphorylation of tyrosine residues of the EGFR was not observed (Figure 4B). However, AG1478 did not inhibit TF-3-induced ubiquitination and EGFR down-regulation. These results indicate that TF-3-induced EGFR down-regulation does not require tyrosine receptor kinase phosphorylation and activation.

Levkowitz *et al.* [21] showed that one of the mutants of the EGFR, whose tyrosine 1045 was replaced with phenylalanine, lost the ability to undergo ubiquitination *in vitro*. We examined TF-3-induced EGFR down-regulation in HEK 293 cells transiently overexpressing a wild-type (WT) or a Y1045F mutant of the EGFR (Figure 5). TF-3 induced EGFR down-regulation and

ubiquitination in Y1045F mutants as well as WT cells indicating that TF-3-induced EGFR down-regulation and ubiquitination do not depend on phosphorylation of tyrosine 1045 of the EGFR.

Effects of TF-3 on the EGFR Signaling Pathway

To study the biological significance of EGFR down-regulation induced by TF-3, we investigated the effect of TF-3 on the EGFR signaling pathway in JB6 Cl41 cells, which is a well-developed cell culture system for studying genetic susceptibility to anchorage-independent cell transformation, promotion and progression [26,27]. TF-3 induced EGFR down-regulation in a time-dependent manner in JB6 Cl41 cells (Figure 6A). Pretreatment with TF-3 for 1 h inhibited EGF-induced phosphorylation of the EGFR (Figure 6B) and ERKs, which are downstream signaling components of the EGFR [31] (Figure 6C). TF-3 also suppressed AP-1 activation (Figure 6D) in a dose-dependent manner.

TF-3 Inhibits EGF-Induced Anchorage-Independent Cell Transformation

Finally, we performed the anchorage-independent transformation assay using TF-3. TF-3 (10 or 20 μ M) significantly inhibited EGF-induced anchorage-independent growth of JB6 Cl41 cells (Figure 7). Taken together, TF-3 seems to show chemopreventive effects through the inhibition of the EGFR signaling pathway resulting from EGFR down-regulation.

DISCUSSION

The EGFR has been recognized as a convergence point for diverse signal transduction pathways [32]. EGF binding to the EGFR is known to induce EGFR down-regulation [28] and the molecular mechanism has been well-described [29]. This process is believed to require EGFR tyrosine kinase activation followed by ubiquitination of the receptor. Levkowitz *et al.* [10,21] reported that when EGF binds to the extracellular portion of the EGFR, c-Cbl, a ubiquitin ligase, binds to tyrosine 1045 of the EGFR, which is one of the autophosphorylation sites of the receptor tyrosine kinase. Then, the cytoplasmic portion of the EGFR is degraded by the proteasome, followed by lysosomal hydrolysis [29]. The EGFR is commonly overexpressed in many human tumors and provides a valid target for anticancer drug development [12]. EGFR specific tyrosine kinase inhibitors and monoclonal antibodies have shown good activity and some EGFR inhibitors have been approved for cancer treatment [33–36].

In this study, we showed that TF-3, one of the components of theaflavins, induces EGFR down-regulation and internalization similar to that induced by EGF. Moreover, TF-3 induced EGFR ubiquitination and receptor tyrosine kinase activation. Therefore, we hypothesized that TF-3 induces down-regulation of the EGFR in a process requiring receptor ubiquitination and tyrosine kinase activation. Interestingly, TF-3-induced EGFR down-regulation was impaired by a proteasome inhibitor, MG132, but not by a receptor tyrosine kinase inhibitor, AG1478, or by the mutation of tyrosine 1045 of the EGFR. Taken together, TF-3-induced EGFR down-regulation appeared to require ubiquitination and degradation by the proteasome, but did not require activation of the receptor tyrosine kinase. These results suggested that TF-3 induces EGFR down-regulation through a pathway distinct from the known EGFR down-regulation induced by EGF binding. We have elucidated portions of the mechanism of EGFR down-regulation induced by TF-3. However, whether TF-3 affects the EGFR directly or indirectly is not yet clear. Liang *et al.* [5] showed that TF-3 inhibits EGF binding to the EGFR. Furthermore, we showed that TF-3 induces EGFR internalization and activates the receptor tyrosine kinase activity suggesting that TF-3 could be acting as a partial agonist. However, we need further study to clarify this issue.

To define the biological significance of the EGFR down-regulation induced by TF-3, we investigated the effects of TF-3 on downstream EGFR signaling pathways. Pretreatment with TF-3 inhibited EGF-induced phosphorylation of the EGFR and ERKs, as well as AP-1 activation. Furthermore, TF-3 inhibited EGF-induced neoplastic transformation in the anchorage-independent transformation assay. These results supported the idea that TF-3 may exert chemopreventive effects through EGFR down-regulation.

Many chemopreventive compounds can induce blocking and suppressing effects. For example, (-)-epigallocatechin-3-gallate (EGCG), a well-characterized chemopreventive compound, not only inhibits the growth factor signaling pathway [5,37], but also induces apoptosis in tumor cells [38]. In this study, we clearly showed that TF-3 inhibits the EGFR signaling pathway. Moreover, theaflavins have been shown to be capable of inducing apoptosis [39-42] and Yang et al. [42] reported that the gallate structure of theaflavins is important in their inhibition of cell growth. In addition, theaflavins are known to be potent inhibitors of the antiapoptotic Bcl-2-family proteins, Bcl-x_L and Bcl-2 [43]. Recently, He *et al.* [44] showed that UVA induces EGFR down-regulation and the down-regulation also does not require receptor tyrosine kinase activity. Furthermore, they showed that UVA-induced receptor down-regulation is dependent on caspase activation, and suggested that the down-regulation may play an important role in apoptosis. One of the biological effects of EGFR down-regulation induced by TF-3 might be apoptosis.

The major process that regulates the amplitude and kinetics of signal transduction by the EGFR is endocytic removal of the active ligand (receptor complexes from the cell surface), and their subsequent sorting to degradation or recycling [29]. Bao *et al.* [45] indicated that protein kinase C (PKC) regulates EGFR recycling back to the cell surface. Intriguingly, Chen *et al.* [46] showed that TF-3 inhibits TPA-induced PKC activity in NIH3T3 cells. Thus EGFR down-regulation induced by TF-3 might also involve inhibition of EGFR recycling mediated by PKC.

In summary, we found that TF-3 induces EGFR down-regulation. This down-regulation is mediated by ubiquitination and the proteasome. However, TF-3-induced down-regulation does not require tyrosine kinase activation. Furthermore, we clearly showed that TF-3 inhibits the EGFR signaling pathway and also EGF-induced anchorage-independent transformation. These results suggest that TF-3 exerts chemopreventive effects through EGFR down-regulation.

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Figure 1.

Theaflavins induce EGFR down-regulation in JB6 Cl41 and A431 EGFR-overexpressing cells. (A) Chemical structure of the various theaflavins. (B) After culturing in serum-free DMEM for 24 h, cells were incubated with individual theaflavins (20 μ M) for 1 h. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted using an antibody against the C-terminus of the EGFR (1005) or β -actin, as indicated. Ctr indicates untreated control.

Figure 2.

TF-3 induces internalization of the EGFR in A431 EGFR-overexpressing cells. (A) Confocal microscope images. After culturing in serum-free DMEM for 24 h, A431 cells were incubated with TF-3 (20 μM) for 1 h. Cells were fixed, permeabilized, and stained with an antibody against the N-terminus of the EGFR (528). This was followed by incubation with Alexa Fluor 568-conjugated anti-mouse IgG and analysis by confocal microscope. (B) A431 cells (3×10^4 cells) were seeded in 96-well plates. After culturing for 24 h, media were replaced with serum-free DMEM and cells were incubated for an additional 24 h. Cells were treated with different concentrations of TF-3 for 1 h. After washing with PBS, cells were incubated with acetic acid (pH 2.7)/NaCl at 4 °C. The relative number of ligand binding sites on the cell surface was determined by incubating cells with biotinylated EGF followed by incubation with streptavidin-HRP and HRP substrate. Absorbance at 415 nm was measured to quantify the biotinylated EGF level. Data are represented as mean $\pm$ S.D. (n = 3). The asterick (*) indicates a significant ($P < 0.05$) decrease in relative number of cell surface receptors in TF-3 treated cells compared to untreated control (lane 1).

Figure 3.

TF-3 induces EGFR down-regulation and ubiquitination in A431 EGFR-overexpressing cells. (A) A431 cells were treated with TF-3 (20 μ M) for the indicated times and proteins were separated by SDS-PAGE. The level of EGFR was determined in whole cell lysates using an antibody against the C-terminus of EGFR (WCL; upper panel). The level of ubiquitinated (Ub) EGFR was determined by immunoprecipitation experiments (lower panel) using an antibody against the N-terminus of the EGFR (528). An antibody to detect total β -actin protein level was used to monitor equal protein loading (middle panel). C indicates untreated control. (B) A431 cells were treated with MG132 (10 μ M), a proteasome inhibitor, or its vehicle, 0.1% DMSO for 1 or 6 h. Cells then were treated with TF-3 (20 μ M) for 1 h in the presence or absence of an inhibitor of protein synthesis, cycloheximide (CHX; 10 μ g/ml). The level of EGFR was determined in whole cell lysates (WCL; upper panels). The level of ubiquitinated (Ub) EGFR was determined by immunoprecipitation (lower panels) experiments using an antibody against the N-terminus of the EGFR (528). An antibody to detect total β -actin protein level was used to monitor equal protein loading (middle panel). IP, immunoprecipitates.

Figure 4.

EGFR down-regulation induced by TF-3 does not require tyrosine kinase activation of the EGFR. (A) TF-3 (upper panel) or EGF (lower panel) induces EGFR autophosphorylation. C indicates untreated control. (B) After treatment with AG1478 (10 μ M), a specific inhibitor of EGFR intrinsic tyrosine kinase activity, or its vehicle, 0.1% DMSO, for 1 h, A431 cells were treated with TF-3 (20 μ M) for 1 h. Proteins in whole cell lysates (WCL) or proteins immunoprecipitated with anti-EGFR (528) were separated by SDS-PAGE and immunoblotted with antibodies to detect phosphorylated tyrosine residues (p-Tyr), EGFR (1005), ubiquitin or β -actin, as indicated. C indicates untreated control. IP, immunoprecipitates.

Figure 5.

Ubiquitination of EGFR induced by TF-3 does not require EGFR tyrosine 1045 phosphorylation. 293 HEK cells were transiently transfected with mock vector, a wild-type EGFR or a tyrosine 1045 mutant (Y1045F) as described in Materials and Methods. Cells were incubated for 36 h at which time media were replaced with serum-free DMEM and cells incubated for an additional 12 h. Cells were then treated with TF-3 (20 μ M) for 1 h. Proteins in whole cell lysates (WCL) or proteins immunoprecipitated with anti-EGFR (528) were separated by SDS-PAGE and immunoblotted with antibodies to detect EGFR (1005) or ubiquitin (Ub) as indicated. IP, immunoprecipitates.

Figure 6.

Effects of TF-3 on the EGFR signaling pathway (A) TF-3 induces EGFR down-regulation in JB6 Cl41 cells in a time-dependent manner. JB6 Cl41 cells were treated with TF-3 (20 μ M) for various times as indicated. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted with antibodies to detect EGFR (1005) or β -actin, as indicated. C indicates untreated control. (B) TF-3 inhibits EGFR phosphorylation induced by EGF. JB6 Cl41 cells were treated with different concentrations of TF-3, or 0.05% ethanol, as a control, for 1 h, and then exposed to EGF (10 ng/ml) for 15 min. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted with antibodies to detect phosphorylation of EGFR, EGFR (1005) or β -actin, as indicated. (C) TF-3 inhibits ERKs phosphorylation induced by EGF. JB6

CI41 cells were treated with TF-3 (20 μ M), or 0.05% ethanol, as a control, for 1 h, and then exposed to EGF (10 ng/ml) for the indicated time. Proteins in whole cell lysates were separated by SDS-PAGE and immunoblotted with antibodies to detect phosphorylation of ERKs or total ERKs proteins, as indicated. C indicates untreated control. (D) Effect of TF-3 on EGF-induced AP-1 activity. JB6 P⁺1-1 cells (8,000 cells) were treated with different concentrations of TF-3 for 1 h, and then exposed to EGF (10 ng/ml). After culturing for 24 h, AP-1 activity was measured by the luciferase activity assay. C indicates untreated control. Data are represented mean $\pm$ S.D. (n=3). Asterick (*) indicates a significant difference ($P < 0.05$) between cells treated with TF-3 and EGF compared to cells treated with EGF alone (lane 2).

Figure 7.

Effect of TF-3 on EGF-induced anchorage-independent transformation in JB6 Cl41 cells. JB6 Cl41 cells (8,000 cells) were exposed simultaneously to 10 ng/ml EGF and different concentrations of TF-3 or 0.05% ethanol, as a control, in 0.33% agar BME containing 10% FBS over 0.5% agar BME containing 10% FBS. C indicates ethanol treated control. Cell colonies were scored after 14 days of incubation at 37 °C in a 5% CO₂ incubator. (A) TF-3 (20 μ M) inhibits EGF-induced anchorage-independent cell transformation. (B) Quantification of total colonies from triplicate samples. Data are represented as mean $\pm$ S.D. Asterick (*) indicates a significant difference ($P < 0.05$) in the number of colonies formed in the presence of TF-3 and EGF versus the number formed in the presence of EGF alone.