Epigallocatechin-3-gallate Inhibits Epidermal Growth Factor Receptor Signaling Pathway

EVIDENCE FOR DIRECT INHIBITION OF ERK1/2 AND AKT KINASES*

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Epidermal growth factor receptor (EGFR) activation is absolutely required for cervical cell proliferation. This suggests that EGFR-inhibitory agents may be of therapeutic value. In the present study, we investigated the effects of epigallocatechin-3-gallate (EGCG), a bioactive green tea polyphenol, on EGFR signaling in cervical cells. EGCG inhibits epidermal growth factor-dependent activation of EGFR, and EGFR-dependent activation of the mitogen-activated protein kinases ERK1/2. EGCG also inhibits EGFR-dependent AKT activity. The EGCG-dependent reduction in ERK and AKT activity is associated with reduced phosphorylation of downstream substrates, including p90RSK, FKHR, and BAD. These changes are associated with increased p53, p21^{WAF-1}, and p27^{KIP-1} levels, reduced cyclin E level, and reduced CDK2 kinase activity. Consistent with these findings, flow cytometry and TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling) staining revealed EGCG-dependent G$_1$ arrest. Moreover, sustained EGCG treatment caused apoptotic cell death. In addition to inhibiting EGFR, cell-free studies demonstrated that EGCG directly inhibits ERK1/2 and AKT, suggesting that EGCG acts simultaneously at multiple levels to inhibit EGF-dependent signaling. Importantly, the EGCG inhibition is selective, as EGCG does not effect the EGFR-dependent activation of JNK. These results suggest that EGCG acts to selectively inhibit multiple EGF-dependent kinases to inhibit cell proliferation.

Cervical cancer is a common cancer among women worldwide. Human papillomavirus (HPV)[1] is implicated as a causative agent in the genesis of this disease (1). High risk HPV subtypes immortalize cervical epithelial cells via the action of two oncogenes, E6 and E7, that enhance cell proliferation by abrogating apoptosis and cell cycle checkpoint function (2). As a result, HPV-immortalized cells acquire increased mitogenic potential and the ability to resist differentiation and apoptosis (3). The epidermal growth factor receptor (EGFR), also referred to as ErbB1, and its downstream effectors play a key role in this process (4–7). EGFR levels and activity increase in cervical cancer (6, 8, 9), along with increased cell proliferation (10, 11), and attenuation of EGFR activity inhibits cervical cell proliferation (12, 13). For this reason, interfering with EGFR function is a focus of anti-cervical cancer therapy. Efforts to inhibit EGFR have focused on decreasing its level and/or activity (14). In addition, downstream EGFR targets, including ERK1/2 and protein kinase B (PKB, also referred to as AKT), are considered viable drug targets. Because cervical disease develops slowly and requires multiple events, the use of dietary chemopreventive agents is an important option for treatment of this disease, which is supported by epidemiologic studies suggesting that modifying the diet can reduce cervical cancer risk (15).

Polyphenols derived from green tea solids are effective chemopreventive agents. (−)Epigallocatechin-3-gallate (EGCG) is the major bioactive polyphenol present in green tea. It possesses anti-oxidant (16), anti-mutagenic (17), anti-proteolytic (18, 19), and anti-proliferative activity (20). In addition, it has been shown to increase p27^{KIP-1} levels, inhibit cyclin activity, and inhibit cell cycle progression (21, 22). In the present study, we examined the ability of EGCG to inhibit EGF-dependent EGFR signaling and downstream MAPK activation. Our studies show that EGCG inhibits EGFR function. Remarkably, this leads to highly specific downstream effects, as only selected downstream EGFR-responsive kinases are reduced in activity. This inhibition ultimately leads to growth cessation and cell apoptosis. Moreover, *in vitro* cell-free studies suggest that EGCG may directly inhibit selected downstream kinases. These studies provide novel new insights into the intracellular mechanism of green tea polyphenol action and suggest that EGCG may be an effective chemopreventive agent for cervical cancer.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assay—Immortalized cervical cells (ECE16-1) and cervical tumor cells (HeLa, Caski, and SiHa) were maintained in complete medium. For experiments, the cells were treated in a defined medium (DM) composed of Dulbecco's modified Eagle's medium:F12 (3:1) supplemented with 1 mg/ml bovine serum albumin, non-essential amino acids, L-glutamine, 5 µg/ml transferrin, 1 nM triiodothyronine, 0.18 nM adenine, 50 µg/ml ascorbic acid, 20 ng/ml EGF, and antibiotics. EGCG was dissolved in sterile water at 50 mM and stored at 4 °C. For proliferation experiments, cells were plated in 24-well plates at 10,000 cells/well in complete medium. Twenty-four

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[1] The abbreviations used are: HPV, human papillomavirus; EGF, epidermal growth factor; EGFR, EGF receptor; DM, defined medium; EGCG, epigallocatechin-3-gallate; FITC, fluorescein isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; PARP, poly(ADP-ribose)polymerase; ERK, extracellular signal-regulated kinase; PKB, protein kinase B; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun NH$_2$-terminal kinase; CDK, cyclin-dependent kinase; FKHR, forkhead transcription factor.

hours after plating, cells were equilibrated in defined medium and treated with EGCG-containing medium on alternate days. For counting, the cells were harvested from triplicate wells with trypsin and counted using a Coulter counter.

Reagents—Dulbecco's modified Eagle's medium, Ham's F-12 medium, medium supplements, and fetal bovine serum were purchased from Invitrogen. Epigallocatechin-3-gallate and anti-β-actin were from Sigma. Histone H1 and pRB was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-activated EGFR antibody (E12120) was obtained from Translab, anti-phosho-ERK1/2 (K-23), anti-ERK1/2 (E-4), anti-CDK2 (M-2), anti-CDK4 (H-22), anti-CDK6 (H-230), anti-cyclin E (M-20), and anti-EGFR (1005) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT (9272), anti-p38 (9212), anti-SAPK/JNK (9252), anti-phospho-AKT (9271), anti-phospho-p38 (9211), anti-phospho-ATF-2 (9225), anti-phospho-ELK-1 (9181), anti-phospho-GSK3 α/β (9331), anti-phospho-BAD (9291), anti-phospho-FKHR (9461), anti-phospho-p90RSK (9344), and anti-phospho-SAPK/JNK (9251) were purchased from Cell Signaling Technologies (Beverley, MA). Anti-cyclin D1, anti-p53, and anti-pRB were from Pharmingen (San Diego, CA). Anti-PARP, anti-p21[WAF-1], anti-p27[KIP-1], and [γ-^{32}P]ATP were purchased from PerkinElmer Life Sciences.

Thymidine Incorporation—Cells were grown for 24 h in defined me-

FIG. 1. **EGCG-dependent suppression of EGFR activation.** Proliferating ECE16-1 cells were plated in standard growth medium. After 24 h, the cells were transferred into EGF-free DM for 16 h to permit EGFR to equilibrate to the cell surface. The cells were then treated for 30 min with the indicated concentrations of EGCG prior to stimulation with 5 ng/ml EGF. After 10 min, whole cell lysates were prepared, and the extracts were electrophoresed at 10,000 cell equivalents/lane and blotted for detection of total and activated (phosphorylated (*P*-)) EGFR. Similar results were observed in each of three separate experiments. The histogram in each panel indicates the relative band intensity, in arbitrary density units, derived from densitometry scans.

dium containing various concentrations of EGCG and then shifted to defined medium containing 1 μCi of [^{3}H]thymidine (61 Ci/mmol)/ml for 2 h. The cells were thoroughly washed with Hanks' balanced salt solution followed by a single wash with 5% trichloroacetic acid, dissolved in 2 N NaOH, and counted in a liquid scintillation counter.
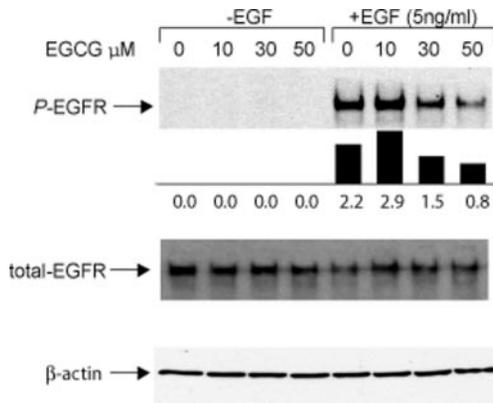
Cell Cycle Analysis—EGCG-treated cells were harvested, fixed with methanol, washed, treated with RNase A, and stained for DNA with propidium iodide as described previously (23). The fraction of cells present in each cell cycle phase (G_0/G_1, S, G_2 + M), as well as apoptotic cells, was determined by mathematical modeling of DNA histograms representing 10,000 events or more. The modeling software was Modfit from Verity House (Topsham, ME). The model components were based on normal distributions for Go/G_1 and G_2 + M and a single broadened trapezoid for S-phase as described previously (23, 24).

Apoptosis Assay—Cells were seeded in growth medium at 1×10^5 cells/35-mm dish, allowed to attach overnight, and then treated with 5–50 μM EGCG in DM for 0–48 h. Cells were harvested with trypsin, washed once, and resuspended in Dulbecco's modified Eagle's medium. To detect annexin V, cells were incubated with annexin V-FITC for 20 min at 37 °C and then washed to remove unbound annexin. Propidium iodide was added, and the cells were incubated for an additional 30 min. The labeled cells were analyzed by flow cytometry. Cells that showed FITC staining were designated as apoptotic, whereas the double stained cells were designated as post-apoptotic.
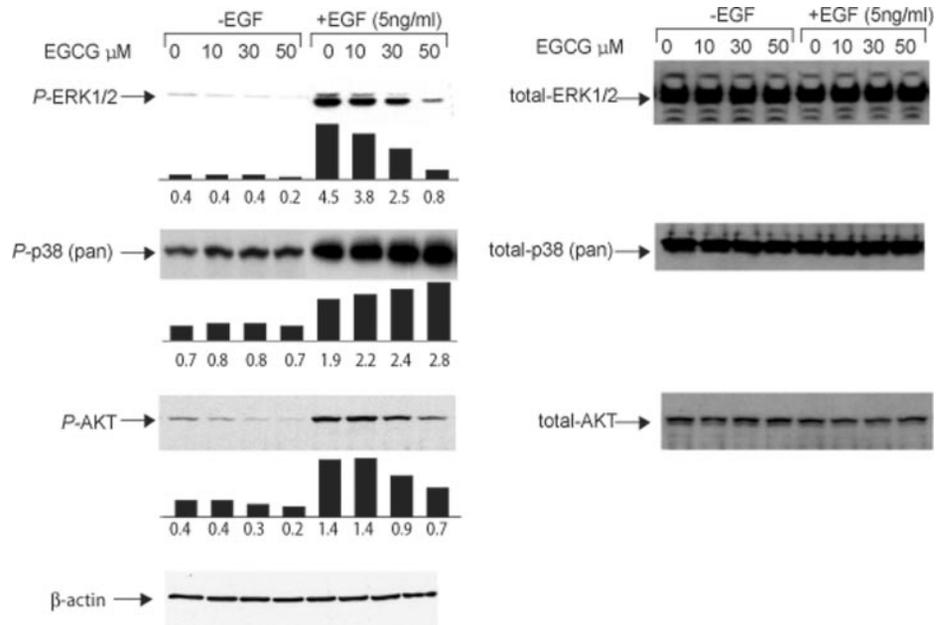
TUNEL Assay—A TUNEL assay was performed using ApopTag (Intergen, Purchase, NY). Cells were seeded at $1–4 \times 10^4$ cells/well in 12-well cluster dishes and allowed to attach overnight. The cells were switched to DM for 24 h and then treated with EGCG for 0–48 h. The cells were then fixed with 1% paraformaldehyde, washed with phosphate-buffered saline, and permeabilized with pre-chilled (-20 °C) ethanol:acetic acid (2:1 v/v). After further washing in phosphate-buffered saline, the cells were incubated in DNA labeling solution (enzymatic incorporation of FITC-UTP into stand breaks) for 1 h at 37 °C and washed with stop/wash buffer. The cells were then mounted in Anti-Fade® (Molecular Probes) and examined with a fluorescence microscope.

Immunoblot—Total cells extracts were prepared in Laemmli sample buffer and electrophoresed at 10,000 cell equivalents/lane on denaturing acrylamide gels. The separated proteins were transferred to polyvinylidene difluoride membranes and incubated with primary antibody. Binding of the primary antibody was detected using a horseradish peroxidase-conjugated secondary antibody and chemiluminescent substrate (West Pico, Pierce). Densitometric analysis was performed using a Bio-Rad FluorS.
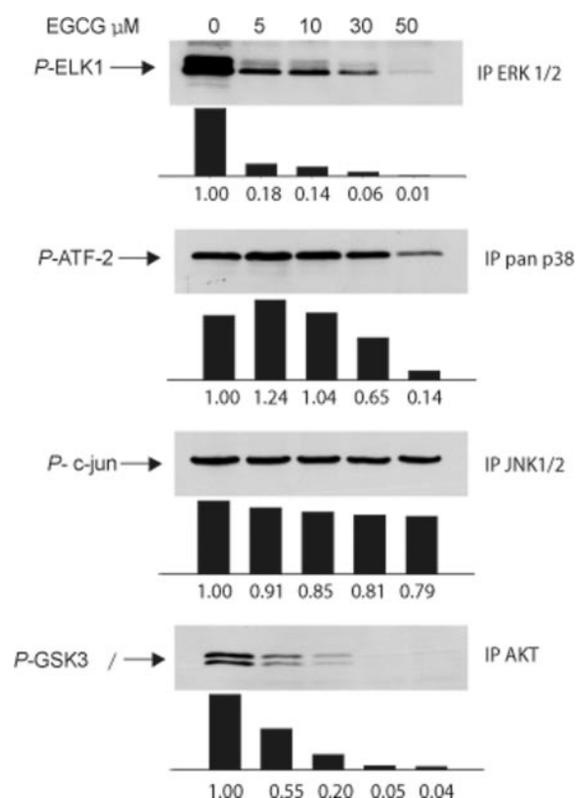
Kinase Immunoprecipitation—Cells were lysed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 50 mM NaF, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin, bestatin, pepstatin A, leupeptin, and E64. Following lysis, protein was estimated using a modified Folin-Lowry protocol (Bio-Rad), and 250 μg of protein lysate was used per kinase reaction. One microgram of

FIG. 2. **EGCG effects on EGFR-dependent downstream kinases.** Lysates prepared from cells treated and harvested as described in the legend to Fig. 1 were blotted and probed for total and phosphorylated (*P*-) ERK1/2, AKT, and p38. The data presented are representative of a minimum of three separate experiments as described in the legend to Fig. 1.

FIG. 3. **EGCG directly inhibit ERK1/2 and AKT kinase activity.** ECE16-1 cells were plated in 100-mm dishes and allowed to reach 60–70% confluence. The cells were transferred into EGF-free DM for 16 h to optimize the level of surface EGFR and bring the system to a stable low level of activity. These cells were then stimulated with 10 ng/ml EGF for 30 min to activate endogenous kinases. Cell lysates were prepared, and the indicated kinases (ERK1/2, p38, JNK1/2, and AKT) were immunoprecipitated (*IP*) (250 μg of protein equivalents of lysate/reaction) and used in kinase assays with the appropriate protein substrate and ATP in the presence of 0–50 μM EGCG for 30 min at 30 °C. The reactions were stopped by the addition of SDS-PAGE sample buffer. Equivalent amounts of reaction mixture was electrophoresed and blotted. The incorporation of phosphate into the protein substrate was detected using the appropriate phospho-specific antibody. The immunoblots depicted correspond to ERK1/2 (*P*-ELK1), p38 (*P*-ATF-2), JNK1/2 (*P*-c-jun), and AKT (*P*-GSK3 α/β). The total level of each kinase was constant in each reaction (not shown).

primary antibody was added to each sample followed by incubation for 2 h at 4 °C. Protein A/G beads were added, and samples were rotated at 4 °C overnight. The beads were washed four times with lysis buffer followed by two washes with kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCI2, 2.5 mM EGTA, I mM dithiothreitol, 5 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF). Cyclin-dependent protein kinase activity was measured by the incorporation of [^{32}P]ATP into protein substrate. The immunoprecipitated kinase bound to the A/G beads are incubated in kinase buffer supplemented with 20 pM ATP, 1 μg of protein substrate, and 5 μCi of [γ-^{32}P]ATP. Reactions were stopped by the addition of an equal volume of 2$\times$ Laemmli buffer followed by SDS-PAGE and exposure to film. MAPK and ATK activities were measured in nonradioactive kinase assays using kits purchased from Cell Signaling Technologies. Washed beads from the immunoprecipitation were incubated for 30 min at 30 °C with 20 pM ATP and 1 μg of protein substrate, and the extent of phosphorylation of substrate was determined using the appropriate phospho-specific antibody followed by chemiluminescent detection.

RESULTS

EGCG Inhibits EGFR Activation—EGFR activity is required for growth of ECE16-1 cells (12, 13). To determine whether EGCG effects EGFR function, ECE16-1 cells were cultured overnight in the absence of EGF stimulation. The cells were then treated with 0–50 μM EGCG for 30 min followed by 5 ng/ml EGF for 10 min. The *upper panel* in Fig. 1 shows a
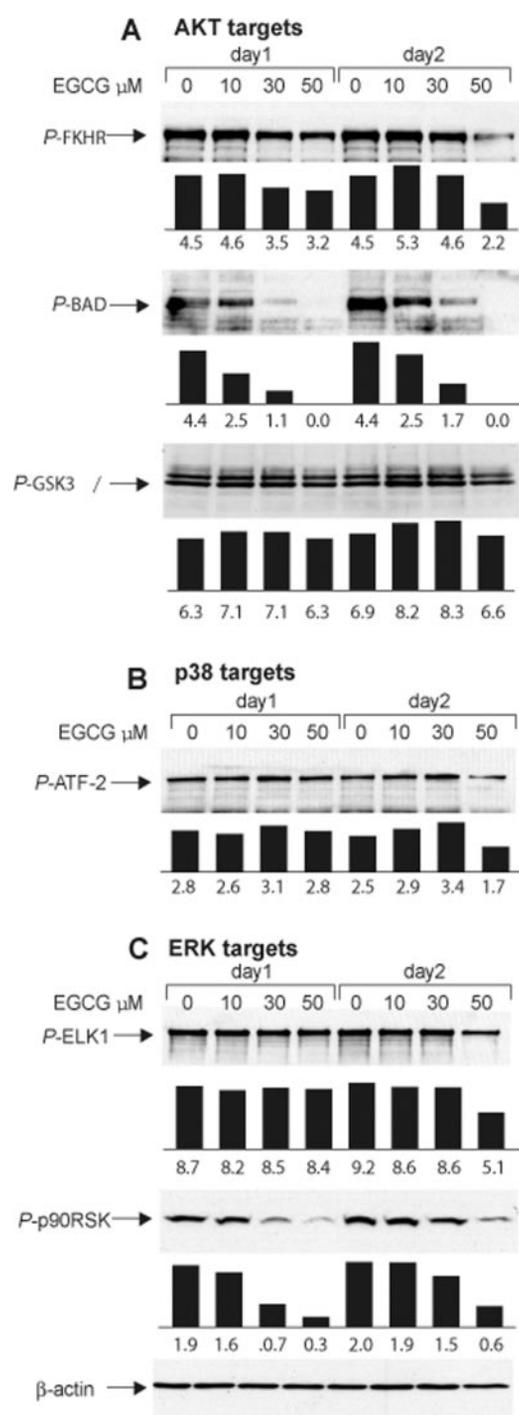
FIG. 4. **EGCG inhibits phosphorylation of FKHR, BAD, and p90^{RSK}.** ECE16-1 cells were plated at 20,000 cells/well in growth medium. After 24 h they were transferred to a defined medium with EGF. The cells were then treated with the indicated concentrations of EGCG for 24 or 48 h in defined medium. Whole cell lysates were prepared, electrophoresed at 10,000 cell equivalents/lane, and blotted for detection of activated (phosphorylated (*P*-)) FKHR, BAD, GSK3 α/β, ATF-2, ELK-1, and p90^{RSK}. The total level of each protein was not altered by treatment (not shown). The β-actin level was monitored to confirm appropriate gel loading.

marked activation of EGFR (*P*-EGFR) in EGF-treated cells. EGCG treatment results in a concentration-dependent reduction in this activity. Activity is reduced by 65% in cells treated with 50 μM EGCG. Total EGFR level is not affected by EGCG treatment (Fig. 1).

EGCG Suppresses EGFR-dependent Activation of ERK1/2, and AKT—We next evaluated whether the EGCG-dependent

EGCG Inhibits EGFR Signaling
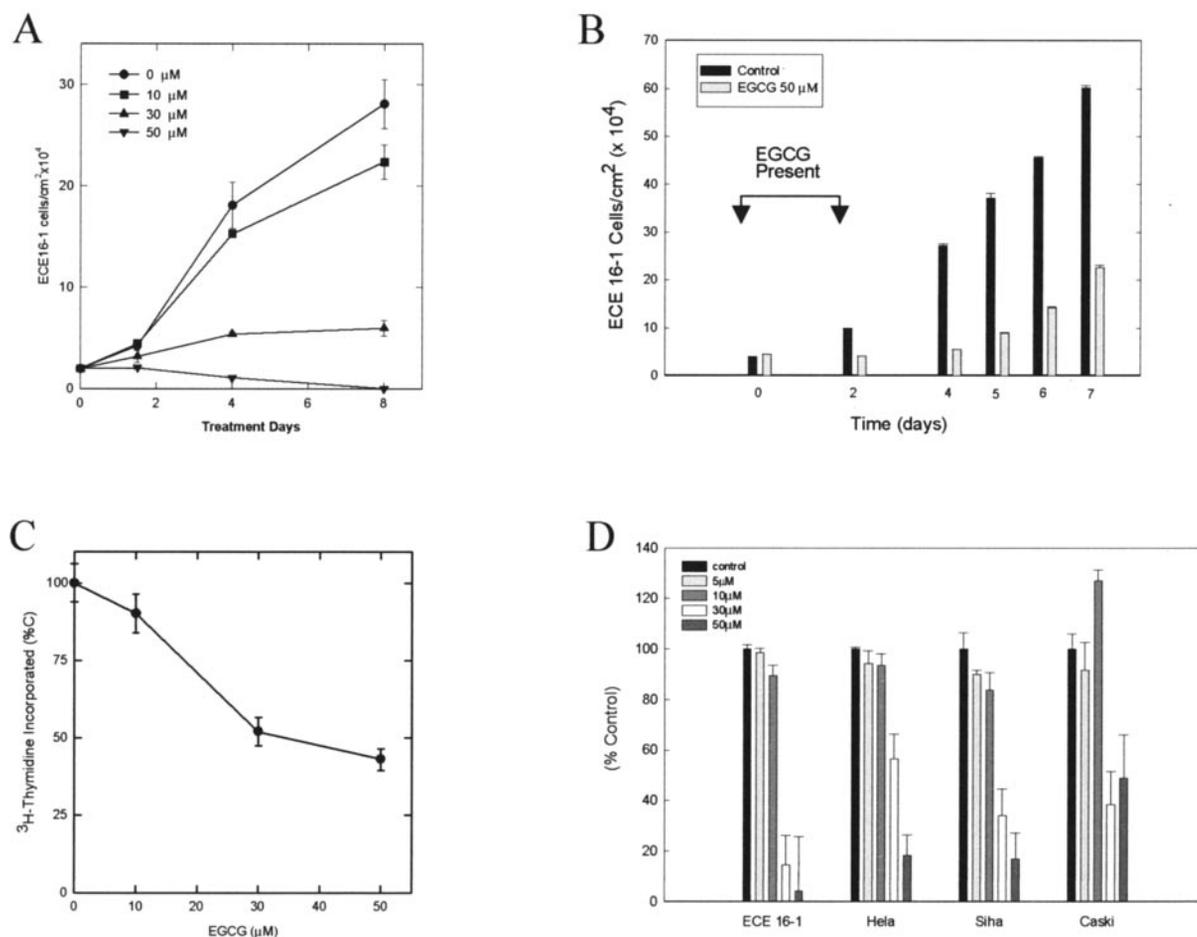
FIG. 5. **EGCG treatment suppresses cell number.** *A*, ECE16-1 cell growth arrest time course. ECE16-1cells were seeded in 24-well clusters at $1–2 \times 10^4$ cells/dish and treated with the indicated concentrations of EGCG; triplicate wells were harvested and counted on days 2, 4, and 8. *B*, EGCG growth inhibition is reversible. ECE16-1 cells were treated for 48 h with either vehicle or 50 μM EGCG, washed extensively, and cultured in medium devoid of EGCG. Following a lag in proliferation, EGCG-treated ECE16-1 cells proliferate with growth kinetics similar to vehicle-treated cells. *C*, [^{3}H]thymidine incorporation in ECE16-1 cells pretreated with the indicated concentrations of EGCG for 24 h. ECE16-1 cells show a dose-dependent decrease in proliferation with ~50% lower incorporation of [^{3}H]thymidine in cells treated with 50 μM EGCG. *D*, EGCG inhibits proliferation of other cervical cell lines. Cervical cell lines were treated as described in *A* for 4 days and then harvested, and triplicate wells were counted. All data are representative of a minimum of three independent experiments. Each experiment was counted in triplicate.

reduction in EGFR activity is associated with reduced downstream kinase activity. ECE16-1 cells display a characteristic EGF-dependent activation of ERK, JNK, p38, and AKT (12). Treatment with 50 μM EGCG reduced EGF-dependent ERK1/2 activity by 83% and AKT activity by 50% (Fig. 2). In contrast, EGCG does not reduce *P*-JNK1/2 level (not shown), and EGCG produces a slight increase in p38 activity. The selective inhibition of ERK1/2 and AKT activity is noteworthy because these kinases regulate cell proliferation and survival (25–27). The activation of p38 is also important given its role in cell differentiation (28). Neither EGF nor EGCG treatment altered the absolute level of ERK, p38, or AKT (Fig. 2). EGF-dependent activation of EGFR results in the biphasic activation of ERK in ECE16-1 cells with peak activity at 10 min and 24 h (12). EGCG inhibited both peaks of ERK activation (not shown).

EGCG Directly Inhibits ERK1/2 and AKT Activity—We next examined whether downstream kinases are directly effected by EGCG. Extracts were prepared from EGF-stimulated cells and various downstream kinases were collected by immunoprecipitation. The precipitated kinases were then incubated in *in vitro* kinase reactions in the presence of 0–50 μM EGCG. As shown in Fig. 3, low concentrations of EGCG (5 μM) caused a substantial reduction in AKT and ERK1/2 activity. Higher EGCG concentrations inhibit p38 MAPK. In contrast, JNK1/2 activity was not altered.

EGCG Inhibits Protein Phosphorylation Downstream of AKT—AKT targets the forkhead transcription factor, FKHR, the BCL-2 family member BAD, and GSK3α/β. As seen in Fig. 4*A*, treating cells with EGCG caused reduced FKHR and BAD phosphorylation. In contrast, GSK3α/β phosphorylation was not significantly altered. Phosphorylation of the p38 MAPK downstream target, ATF-2, was enhanced at intermediate EGCG concentrations (Fig. 4*B*). Phosphorylation of ELK, the ERK1/2 target, was slightly reduced. However, the activity of the ERK target, p90RSK, was markedly inhibited by EGCG (Fig. 4*C*). These findings indicate that EGCG selectively inhibits phosphorylation of specific substrates.

EGCG Treatment Inhibits Cell Proliferation—Reduced MAPK and PKB/Akt activity is frequently associated with reduced cell numbers (29, 30). It was therefore of interest to determine whether EGCG would suppress cell growth or increase cell death. As shown in Fig. 5*A*, EGCG produced a concentration-dependent reduction in ECE16-1 cell number that was detected as early as 36 h after initiation of treatment. To assess the reversibility of the EGCG-dependent response, cells were treated with or without 50 μM EGCG. After a 48-h treatment, the cells were transferred to EGCG-free medium, and cell growth was continued. As shown in Fig. 5*B*, the removal of EGCG restores cell proliferation to a rate similar to that observed in untreated cultures. A similar pattern of recov-
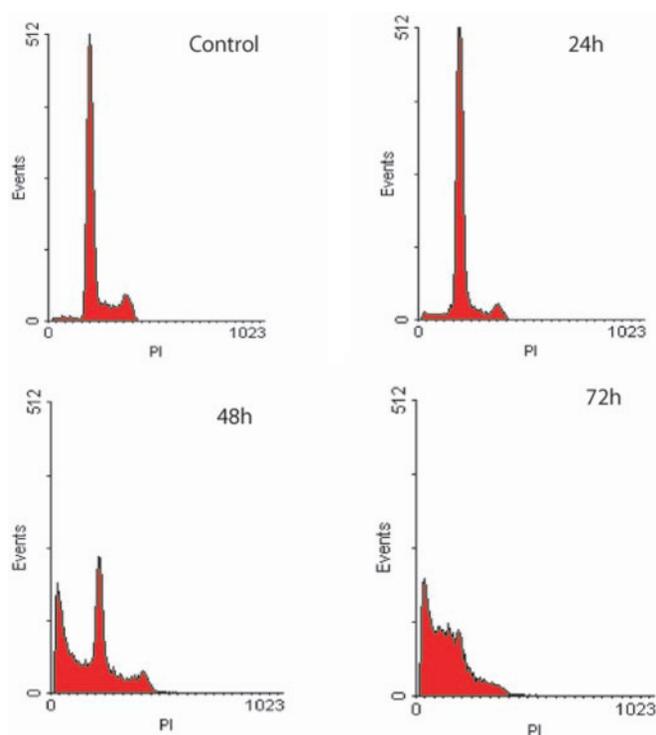
FIG. 6. **EGCG effects on cell cycle in ECE16-1 cells.** ECE16-1 cells were treated with vehicle or 50 μM EGCG-supplemented medium for 1–3 days. After treatment the cells were harvested with trypsin, washed, fixed with methanol, stained, and analyzed by flow cytometry as described under "Materials and Methods." The data were analyzed using the Modfit modeling program.

ery was observed for HeLa cells (not shown). DNA synthesis was monitored as a second index of cell proliferation. ECE16-1 cells were treated for 24 h with EGCG and then pulsed with [³H]thymidine prior to harvest. As seen in Fig 5*C*, EGCG treatment produces a concentration-dependent decrease in [³H]thymidine incorporation. To determine whether these effects can be generalized to other cervical cell lines, we treated HeLa, CaSki, and SiHa cells with EGCG. As shown in Fig. 5*D*, all lines were inhibited by EGCG treatment.

EGCG Treatment Increases Cell Death—Reduced cell numbers could be the result of diminished cell proliferation or increased cell death. As an initial approach to identifying the mechanism of cell death, we treated ECE16-1 cells with EGCG and then analyzed them by flow cytometry. Fig. 6 shows that EGCG treatment for 24 h causes accumulation of cells in G_1 and treatment for 48 and 72 h causes accumulation of sub-G_1 cells. This accumulation of sub-G_1 cells is associated with increased accumulation of surface annexin (Fig. 7). Untreated ECE16-1 cells are propidium iodide-positive (4–8%), annexin-positive (1–3%), or positive for both (4–10%). Treatment with concentrations of EGCG ≤ 10 μM does not alter this distribution. In contrast, treatment with 30 and 50 μM EGCG results in a marked increase in annexin- and propidium iodide-positive cells, indicating an increase in advanced stage apoptosis (Fig. 7, *C* and *D*). EGCG-dependent induction of annexin binding was also observed in HeLa and SiHa cells (Fig. 7*E*).

We used a TUNEL assay as a third method of evaluating apoptosis. Staining ECE16-1 cells following EGCG treatment revealed numerous TUNEL-positive cells (Fig. 8*A*). In contrast, untreated ECE16-1 cells were not stained. In addition, treatment of ECE16-1 cells with EGCG resulted in enhanced levels of cleaved (89 kDa) PARP. TUNEL-positive cells were also observed in EGCG-treated CaSki, HeLa, and SiHa cells (Fig. 8*B*).

EGCG Perturbs Cell Cycle Regulators—As shown in Fig. 9*B*, EGCG increases p53 but not pRB level. The increase in p53 protein expression was associated with an increased p21$^{\text{WAF-1}}$ level and diminished cyclin E level (Fig. 9*A*). The p27 level was also increased, but CDK2, CDK4, and cyclin D1 levels did not change (Fig. 9*A*). We also measured CDK activity. As shown in Fig. 10, EGCG treatment reduced the CDK2 kinase activity as measured by the ability to phosphorylate H1 histone. In contrast, CDK4 and CDK6 activity were not changed.

DISCUSSION

ECE16-1 cells are immortalized cervical cells, which mimic human disease in that they over-express EGFR, grow at a fast rate, and have lost the ability to terminally differentiate. We have previously shown that proliferation of these cells requires EGF (13) and that proliferation is inhibited by blocking EGFR signaling or reducing EGFR level (12, 13). Our present studies show that EGCG inhibits EGF-mediated EGFR activation. This inhibition is associated with reduced activation of ERK1/2 and AKT. The inhibition of EGFR, AKT, and ERK1/2 is significant, as these kinases play a key role in cervical cancer cell proliferation (6, 31–33). Cell-free experiments demonstrate that EGCG can directly inhibit AKT and ERK1/2 activity. Thus, EGCG may inhibit cell proliferation by direct interaction with kinases at multiple levels in the signaling cascade. This suggestion is consistent with recent reports in other systems (34–36).

An interesting feature of this regulation is the selectivity. ERK1/2, p38, JNK1/2, and AKT/PKB are all known targets of EGFR (29, 37). However, only ERK1/2 and AKT/PKB activity is inhibited by EGCG *in vivo*. In contrast, p38 is slightly activated, and JNK1/2 activity does not change. In particular, phosphorylation of several kinases and downstream targets in the EGFR and AKT/PKB pathways is strongly suppressed by EGCG treatment. These targets include EGFR, AKT/PKB, FKHR, and BAD. However, the *in vivo* activity of another kinase in this cascade, GSK3α/β, is not affected. This selectivity is likely to be important, as FKHR and BAD regulate apoptosis. Dephosphorylated FKHR translocates to the nucleus and increases expression of BIM and FAS ligand (both pro-apoptotic regulators (38)), whereas dephosphorylated BAD sequesters and inactivates the anti-apoptotic protein BCL-X$_\text{L}$ (39). These results suggest that EGCG treatment predisposes the cells to apoptosis. Recent reports also demonstrate that AKT influences p53 level by altering nuclear mdm2 expression (40, 41). Moreover AKT also decreases the p27 level (42–45). These findings predict that inhibiting AKT activity should increase p53 and p27 levels. Indeed, our results indicate that EGCG treatment produces increased p53 and p27. Increased p53 and p27 levels are associated with the G_1 arrest observed when EGFR expression is inhibited (39, 46, 47). On balance, these findings suggest a pathway in which EGCG inhibition of EGFR leads to reduced AKT/PKB, FKHR, and BAD activity, increased levels of p53 and p27, and cell cycle arrest.

Activity is also selectively altered in the ERK1/2 cascade. Sustained activation of ERK1/2 is necessary for cell survival and cell proliferation (48). Our studies show that ERK1/2 activity is reduced. However, the activity of ERK1/2 downstream targets is differentially regulated. For example, ELK1 activity is not changed in EGCG-treated cells, but activity of another ERK1/2 target, p90$^{\text{RSK}}$, is markedly reduced by EGCG treatment. p90$^{\text{RSK}}$ phosphorylates the transcriptional co-activators p300 and CBP (cAMP-response element-binding protein (CREB)-binding protein) (49). These acetyltransferases associate with transcriptional regulators to promote expression of genes required for S-phase progression (50). Based on these functions, we propose that reduced p90RSK activity may con-
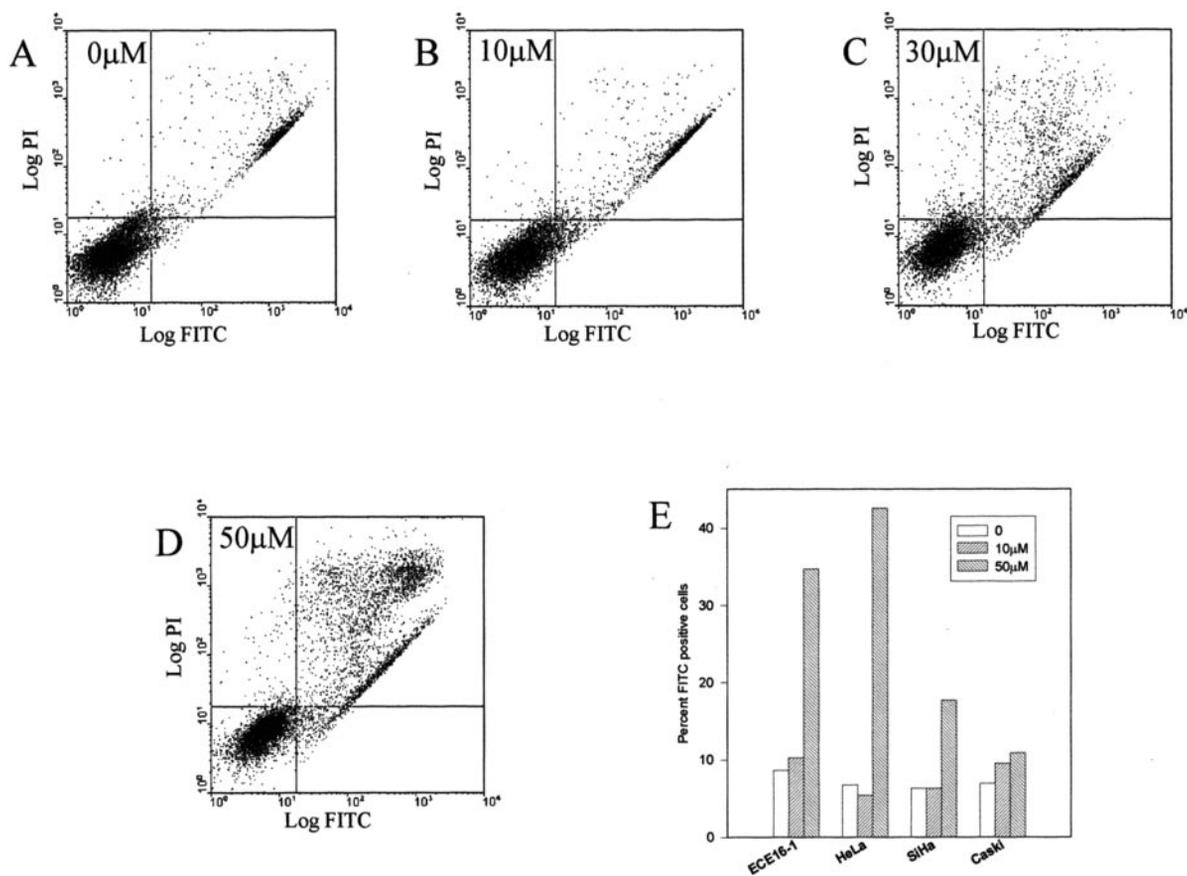
FIG. 7. **EGCG-dependent increase in phosphatidylserine level in the outer plasma membrane leaflet.** *A–D*, phosphatidylserine levels were detected by annexin V-FITC binding. ECE16-1 cells, treated with 0–50 μM EGCG for 48 h and stained with FITC-conjugated annexin V and propidium iodide (*PI*). Cells that are annexin-positive and propidium iodide-negative (*upper left corner* of *lower right quadrant*) are representative of early apoptotic cells. Cells strongly positive for both annexin and propidium iodide (*above* the *diagonal axis* of the *upper right quadrant*) are late apoptotic/necrotic cells. *E*, summary of dot-plot results for five independent experiments expressed as percent annexin-positive cells.
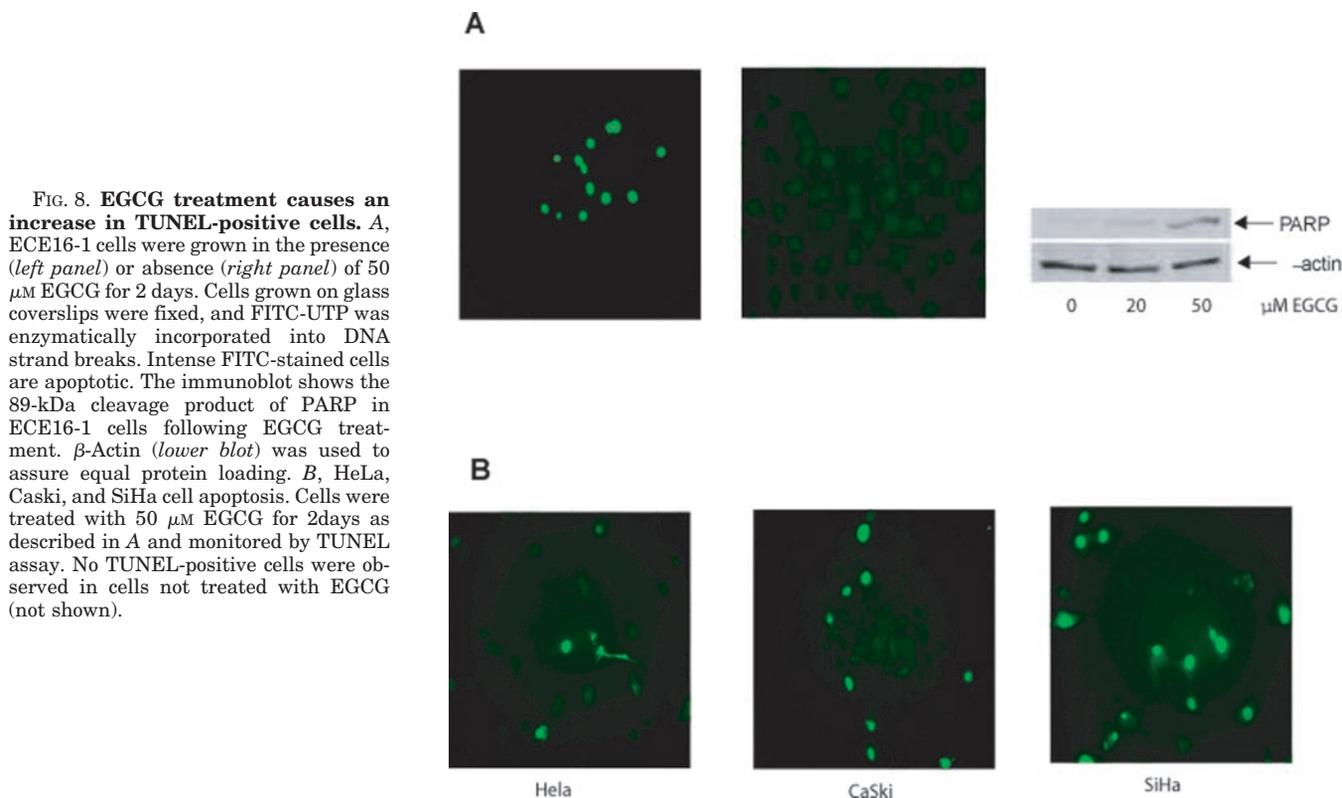
FIG. 8. **EGCG treatment causes an increase in TUNEL-positive cells.** *A*, ECE16-1 cells were grown in the presence (*left panel*) or absence (*right panel*) of 50 μM EGCG for 2 days. Cells grown on glass coverslips were fixed, and FITC-UTP was enzymatically incorporated into DNA strand breaks. Intense FITC-stained cells are apoptotic. The immunoblot shows the 89-kDa cleavage product of PARP in ECE16-1 cells following EGCG treatment. β-Actin (*lower blot*) was used to assure equal protein loading. *B*, HeLa, Caski, and SiHa cell apoptosis. Cells were treated with 50 μM EGCG for 2 days as described in *A* and monitored by TUNEL assay. No TUNEL-positive cells were observed in cells not treated with EGCG (not shown).
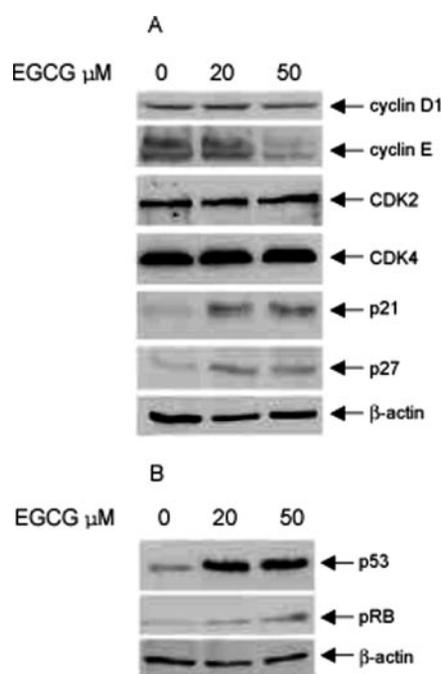
A

EGCG μM   0   20   50

← cyclin D1
← cyclin E
← CDK2
← CDK4
← p21
← p27
← β-actin

B

EGCG μM   0   20   50

← p53
← pRB
← β-actin

FIG. 9. **EGCG effects on cell cycle regulatory protein level.** ECE16-1 cells were treated with 0, 20, or 50 μM EGCG for 24 h. Total cell lysates were prepared and analyzed for expression of p53 and pRB (*A*) and various cell cycle kinases and their activators and inhibitors (*B*). The β-actin level was monitored to assure consistent loading.

EGCG μM   0   10   30   50

IP-CDK2 /H1 peptide

IP-CDK4/ RB peptide

IP-CDK6/ RB peptide

FIG. 10. **EGCG inhibits CDK2 activity.** ECE16-1 cells were plated in 100-mm dishes and allowed to reach 60–70% confluence, at which time they were treated with various concentrations of EGCG for 24 h. Cell lysates were prepared, and the indicated kinases (CDK2, CDK4, and CDK6) were immunoprecipitated (*IP*) (500 μg of protein equivalents of lysate/reaction) and used in kinase assays with the appropriate protein substrate and ATP. The reactions were stopped by the addition of SDS-PAGE sample buffer.

tribute to the cessation of proliferation.

EGCG and Cell Proliferation/Apoptosis—Our results show that the effects of EGCG can be reversed after short term treatment. However, long term treatment leads to an irreversible increase in sub-G_1 cell numbers, increased numbers of annexin- and propidium iodide-positive cells, increased TUNEL staining, and increased caspase and PARP cleavage. These findings argue for a temporal progression wherein EGCG initially reversibly inhibits proliferation, as manifested, for example, by increased levels of p21, p27, and p53, and that prolonged EGCG treatment results in a irreversible apoptotic commitment as evidenced by increased TUNEL staining, PARP cleavage, etc. As ERK1/2 and AKT/PKB signaling is reported to be required for cell survival (25, 51), we hypothesized that the transition from growth inhibition to apoptosis is due to a sustained EGCG-dependent inhibition of the ERK1/2 and AKT/PKB signaling cascades.

Selective Inhibition of Kinase Activity by EGCG—As noted above, not all kinases are inhibited by EGCG. Neither JNK1/2 nor p38 activity is inhibited by EGCG. JNK1/2 activity remains unchanged, suggesting that it does not mediate the EGCG-associated responses. p38 is somewhat enhanced following treatment. This may be important, as p38 is known to enhance differentiation in surface epithelia and may contribute to cell death (52, 53). As the four p38 isoforms are known to have differential effects (52, 53), additional studies will be required to identify which form is modified by EGCG in our system. Moreover, as noted above, phosphorylation of only selected targets downstream of ERK1/2 is suppressed by EGCG. ELK1 activity is not regulated, whereas p90[RSK] activity is markedly suppressed. Likewise, among AKT/PKB targets, FKHR and BAD activity are suppressed, but GSK3α/β activity is not regulated. This suggests that the net effect of EGCG treatment is the integration of a complex set of effects on intracellular kinase activity.

Our results indicate that EGCG inhibits EGFR, the initial kinase in the EGF signaling cascade. Based on this finding, it
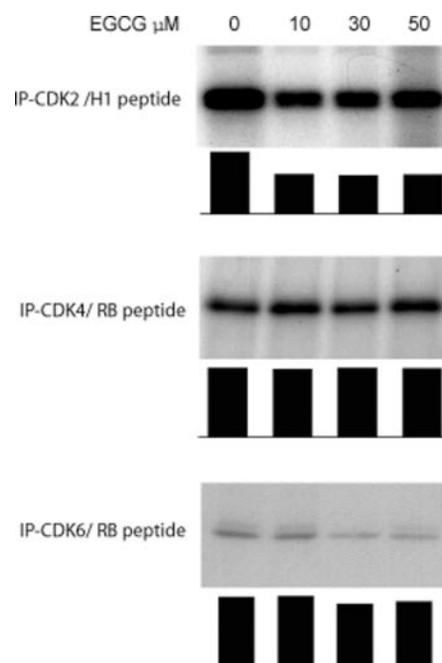
would be logical to assume that the reduction in activity of kinases downstream of EGFR is solely due to the reduction in EGFR activity. However, *in vitro* cell-free assays suggest that the regulation may be more complex. Direct incubation of activated ERK1/2 or AKT/PKB with EGCG inhibits activity in an EGCG concentration-dependent manner. p38 activity is also reduced, but JNK1/2 activity is not regulated in this type of assay. This suggests, first, that the inhibition of activity is specific, as not all kinases are inhibited. Second, it is noteworthy that in most cases suppression of the enzymes in the cell-free *in vitro* assay matches the response in intact cells (ERK1/2 and AKT/PKB). Thus, it may be that ERK1/2 and AKT/PKB are inactivated in the cell by dual effects of EGCG, the suppression of incoming EGF-associated stimulation and direct inhibition of these kinases. However, some kinases (*i.e.* p38) are inhibited by EGCG in the cell-free system but not by EGCG treatment of intact cells. These findings point to the complexity of this regulation, suggesting that it is incorrect to assume that inactivation of the initial kinase in a cascade will result in inactivation of all downstream responses.

EGCG and the Human Papillomavirus E6/E7 Oncoproteins—Human papillomavirus is a major etiological agent in the genesis of cervical cancer. Immortalization of normal cervical cells by HPV is the initial step in this process. This immortalization requires the action of the HPV oncoproteins, E6, which reduces p53 level, and E7, which increases cyclin E expression (54). In principle, it would seem possible that HPV-immortalized cells might overcome the anti-survival effects of EGCG due to the effects of E6 and E7. However, the fact that EGCG treatment increases CDK inhibitor levels despite the E6/E7-positive background suggests that EGCG may be an effective therapeutic/preventive agent in cervical cancer.

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EGCG Inhibits EGFR Signaling

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Mechanisms of Signal Transduction:

Epigallocatechin-3-gallate Inhibits Epidermal Growth Factor Receptor Signaling Pathway: EVIDENCE FOR DIRECT INHIBITION OF ERK1/2 AND AKT KINASES

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