Apigenin Inhibition of Involucrin Gene Expression Is Associated with a Specific Reduction in Phosphorylation of Protein Kinase Cδ Tyr311*

Sivapraokam Balasubramanian, Ling Zhu, and Richard L. Eckert

From the Departments of Physiology and Biophysics, Dermatology, Biochemistry, Reproductive Biology, and Oncology, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4970

Received for publication, June 5, 2006, and in revised form, September 7, 2006. Published, JBC Papers in Press, September 18, 2006, DOI 10.1074/jbc.M605368200

Apigenin is a plant-derived flavonoid that has significant promise as a skin cancer chemopreventive agent. In the present study, we examine the mechanism whereby apigenin regulates normal human keratinocyte differentiation. Expression of involucrin (hINV), a marker of keratinocyte differentiation, is increased by differentiating agents via a protein kinase Cδ (PKCδ), Ras, MEKK1, MEK3 cascade that increases AP1 factor level and AP1 factor binding to DNA elements in the hINV promoter. We show that apigenin inhibits this response. Apigenin suppresses the 12-O-tetradecanoylphorbol-13-acetate-dependent increase in AP1 factor expression and binding to the hINV promoter and the increase in hINV promoter activity. Apigenin also inhibits the increase in promoter activity observed following overexpression of PKCδ, constitutively active Ras, or MEKK1. The suppression of PKCδ activity is associated with reduced phosphorylation of PKCδ-Y311. The physiological importance of this phosphorylation event was confirmed by showing that the PKCδ phosphorylation-defective mutant, PKCδ-Y311F, is less able to increase hINV promoter activity. Activation of hINV promoter activity by the green tea polyphenol, (-)-epigallocatechin-3-gallate, is also inhibited by apigenin, suggesting that the two chemopreventive agents can produce opposing actions in keratinocytes. Additional studies show that the apigenin-dependent suppression of differentiation is associated with reduced cell proliferation but that there is no evidence of apoptosis.

Flavonoids are naturally occurring polyphenolic compounds present in plants. Epidemiological studies suggest that flavonoids play an important role in the prevention of disease (1–4). Apigenin (4’,5,7-trihydroxyflavone) is a nonmutagenic flavonoid found in vegetables and fruits, including parsley, onions, wheat sprouts, chamomile, seasonings, tea, and orange (4–7). Apigenin has gained attention due to its health benefits and ability to inhibit cancer development; it is a potent inhibitor of skin, prostate, colon, breast, and thyroid cancer cell proliferation (8–13). Apigenin has been reported to act via several mechanisms, including promotion of cell cycle arrest and apoptosis, inhibition of cell transformation, inhibition of mutagenesis, and suppression of signal transduction, gap junction function, and angiogenesis (14, 15).

Apigenin has also been shown to be active in skin disease models. Topical application of apigenin on skin suppresses ornithine decarboxylase activity and reduces the number and the size of skin tumors induced by chemical carcinogen or UVB exposure (16, 17). In the human-derived immortalized cell line, HaCaT, apigenin treatment suppresses COX-2 expression via a mechanism that involves inhibition of Akt (18). Apigenin also causes G2/M arrest in murine keratinocytes (9, 19), a response that is associated with increased p53 protein stability and increased expression of p21 (8, 20). However, despite the demonstrated activity of apigenin in inhibiting skin cancer and the ability of apigenin to inhibit the proliferation of keratinocyte cell lines, the impact of apigenin treatment on primary human epidermal keratinocytes has not been extensively studied. This is somewhat surprising, since these cells, due to their location on the body surface, are the cell type directly challenged by exposure to UVB and/or environmental carcinogens. Thus, it is important to understand the impact of chemopreventive agent treatment on this cell type.

Keratinocytes undergo a process of differentiation in which proliferating undifferentiated cells, located in the basal epidermal layers, give rise to cells that differentiate as they migrate into the suprabasal layers (21–23). These cells ultimately undergo differentiation-related cell death and are lost from the body surface (24). Cells can also be lost from the epidermis by apoptotic mechanisms. Apoptosis is not frequent in normal epidermis but is observed in epidermis that has been exposed to ultraviolet irradiation (25, 26). Since normal differentiation and apoptosis are two major mechanisms for removing damaged cells from epidermis, it is important to understand how chemopreventive agents impact these processes.

In the present study, we examine the effect of apigenin on normal human keratinocyte signal transduction, apoptosis, and proliferation. We use involucrin gene expression as a marker of skin cell differentiation (27–30). Involucrin expression is restricted to the suprabasal epidermal layers (spinous and granular layers) during normal keratinocyte differentiation and is considered an early marker of the differentiation process (31). Involucrin expression is increased via a novel protein kinase C
Apigenin Regulation of Keratinocyte Survival

Antibodies and Reagents—12-O-Tetradecanoylphorbol-13-acetate (TPA), Me2SO, and apigenin were from Sigma. Apigenin and TPA were prepared as 1000-fold stocks in Me2SO and stored at −70 °C. Keratinocyte serum-free medium (KSFH), trypsin, Hanks’ balanced salt solution, and gentamicin were purchased from Invitrogen. The pGL2-basic plasmid and the chemiluminescent luciferase assay systems were from Promega. [γ-32P]ATP was obtained from PerkinElmer Life Sciences. The human involucrin-specific polyclonal antibody was generated by injecting rabbits with recombinant human involucrin (40). AP1 factor-specific rabbit polyclonal antibodies for Fra-1 (catalog no. sc-605X), Fra-2 (catalog no. sc-171X), c-Fos (catalog no. sc-7202X), FosB (catalog no. sc-48X), c-Jun (catalog no. sc-1694X), JunB (catalog no. sc-46X), and JunD (catalog no. sc-74X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-ERK1/2 (catalog no. M5670), anti-JNK1/2 (catalog no. J4500), anti-p38 (catalog no. M8177), and mouse monoclonal anti-β-actin (catalog no. A5441) were obtained from Sigma. Mouse monoclonal antibodies for PARP (55494) and rabic polyclonal antibody for Bcl-x (catalog no. 610211) were obtained from BD Pharmingen. Rabbit polyclonal antibodies specific for caspase-9 (catalog no. 9502) and caspase-3 (catalog no. 9665) and mouse monoclonal antibody for caspase-8 (catalog no. 9746) were obtained from Cell Signaling. Rabbit polyclonal antibodies for anti-PKCδ (catalog no. SC-937), anti-PKCε (catalog no. SC-214), anti-PKCη (catalog no. SC-215), anti-Ras (catalog no. SC-7181), anti-MEKK1 (catalog no. SC-601), and anti-Bax (catalog no. SC-493) were obtained from Santa Cruz Biotechnology. Anti-PKCδ-Tyr(P)311 (catalog no. 44-950) was obtained from BIOSOURCE. Mouse anti-filaggrin (catalog no. BT-576) and mouse anti-type 1 transglutaminase (catalog no. BT-621) were

(nPKC),2 Ras, MEKK1, MEK3 signaling cascade that regulates AP1 and C/EBP factors interact with specific binding sites on the hINV promoter to increase gene expression (34–36). The present studies show that apigenin inhibits the differentiation-associated increase in hINV gene expression via interference with PKCδ and MAPK signal transduction. In particular, apigenin treatment inhibits phosphorylation of PKCδ tyrosine 311, an event that is required for optimal hINV promoter activity. This action ultimately results in a reduction in AP1 factor expression, leading to a loss of differentiation-associated responses.

(−)-Epigallocatechin-3-gallate (EGCG) is a chemopreventive agent that is effective in reducing the impact of environmental agents on epidermis (37, 38). Our previous studies show that EGCG treatment increases hINV gene expression by activating the MAPK signaling cascade described above (32, 39). Our present studies show that apigenin inhibits this EGCG-dependent increase in keratinocyte differentiation. However, apigenin does not stimulate keratinocyte apoptosis as measured by assessing procaspase cleavage and mitochondrial stability. These findings suggest that chemopreventive agents differentially influence keratinocyte differentiation and apoptosis, a finding that must be considered when these agents are utilized to treat skin cancer.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—12-O-Tetradecanoylphorbol-13-acetate (TPA), Me2SO, and apigenin were from Sigma. Apigenin and TPA were prepared as 1000-fold stocks in Me2SO and stored at −70 °C. Keratinocyte serum-free medium (KSFH), trypsin, Hanks’ balanced salt solution, and gentamicin were purchased from Invitrogen. The pGL2-basic plasmid and the chemiluminescent luciferase assay systems were from Promega. [γ-32P]ATP was obtained from PerkinElmer Life Sciences. The human involucrin-specific polyclonal antibody was generated by injecting rabbits with recombinant human involucrin (40). AP1 factor-specific rabbit polyclonal antibodies for Fra-1 (catalog no. sc-605X), Fra-2 (catalog no. sc-171X), c-Fos (catalog no. sc-7202X), FosB (catalog no. sc-48X), c-Jun (catalog no. sc-1694X), JunB (catalog no. sc-46X), and JunD (catalog no. sc-74X) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit polyclonal anti-ERK1/2 (catalog no. M5670), anti-JNK1/2 (catalog no. J4500), anti-p38 (catalog no. M8177), and mouse monoclonal anti-β-actin (catalog no. A5441) were obtained from Sigma. Mouse monoclonal antibodies for PARP (55494) and rabic polyclonal antibody for Bcl-x (catalog no. 610211) were obtained from BD Pharmingen. Rabbit polyclonal antibodies specific for caspase-9 (catalog no. 9502) and caspase-3 (catalog no. 9665) and mouse monoclonal antibody for caspase-8 (catalog no. 9746) were obtained from Cell Signaling. Rabbit polyclonal antibodies for anti-PKCδ (catalog no. SC-937), anti-PKCε (catalog no. SC-214), anti-PKCη (catalog no. SC-215), anti-Ras (catalog no. SC-7181), anti-MEKK1 (catalog no. SC-601), and anti-Bax (catalog no. SC-493) were obtained from Santa Cruz Biotechnology. Anti-PKCδ-Tyr(P)311 (catalog no. 44-950) was obtained from BIOSOURCE. Mouse anti-filaggrin (catalog no. BT-576) and mouse anti-type 1 transglutaminase (catalog no. BT-621) were

2 The abbreviations used are: nPKC, novel protein kinase C; PKC, protein kinase C; C/EBP, CCAAT enhancer-binding protein; hINV, involucrin; MAPK, mitogen-activated protein kinase; EGCG, (−)-epigallocatechin-3-gallate; KSFH, keratinocyte serum-free medium; PARP, poly(ADP-ribose) polymerase; TPA, 12-O-tetradecanoylphorbol-13-acetate; caRas, constitutively active Ras.
Apigenin Regulation of Keratinocyte Survival

FIGURE 2. Apigenin-dependent regulation requires the proximal regulatory region AP1 site. A, structure of the human involucrin promoter constructs. The nucleotide numbering begins at position −1, adjacent the transcription start site (84, 85). The position of the AP1 factor binding site, AP1-1, and the ets factor (EBS) binding sites (EBS-1 and EBS-2) are indicated. The black rectangle indicates the luciferase, gene and the arrow indicates the transcription start site and direction of transcription. B, 70% confluent keratinocytes were transfected with 2 μg of the indicated involucrin promoter reporter plasmids. The cells were then treated for 24 h with 50 ng of TPA/ml and/or 20 μM apigenin. At 48 h post-transfection, the cells were harvested and lysed for the luciferase activity assay. C, 70% confluent keratinocytes were transfected with 2 μg of pINV-241, pINV-241(AP1-1m), or pINV-241(EBS-2m). The sequence of the AP1-1m and EBS-2m mutants has been reported previously (41). After 24 h, the cells were treated and assayed as above. These plots present the mean ± S.E. compiled from three independent experiments.

obtained from Biomedical Technology, Inc. Rabbit anti-MEK3 (catalog no. SC-961) was obtained from Santa Cruz Biotechnology. Peroxidase-conjugated sheep anti-mouse IgG (catalog no. NA931) and donkey anti-rabbit IgG (catalog no. NA934) secondary antibodies were purchased from Amersham Biosciences. EGCG was obtained from Sigma (catalog no. E4143).

**Plasmids**—The hINV reporter plasmids, encoding various lengths of hINV upstream promoter sequence, fused to the luciferase reporter gene in pGL2-basic, have been previously described (36, 41). PKC expression vectors were kindly provided by Dr. S. Ohno, wild type MEKK1 was obtained from Dr. Dennis Templeton, and constitutively active Ras (RasG12V) was generously provided by Dr. Michael Simonson (41). A plasmid, pPKCδ-EGFP, which encodes PKCδ as a PKCδ-EGFP fusion protein, was used as a template to create PKCδ-Y311F-EGFP using the QuickChange mutagenesis system (Stratagene) and the following primers: 5′-GCTCTGCGAATATTCGAGGATTTGAG (sense) and 5′-CTCAATCCTGGAATATTCGAGCAGAC (antisense). The presence of the mutation that converts tyrosine 311 to phenylalanine was confirmed by DNA sequencing. When convenient, PKCδ-EGFP and PKCδ-Y311F-EGFP are referred to as PKCδ-Y311F and PKCδ-Y311F.

**Cell Culture, Transfection, and hINV Promoter Assay**—Primary human foreskin keratinocytes were cultured as previously described (32, 39). Third passage keratinocytes, grown in 10-cm² dishes, were used when 70% confluent. For transfection, 4 μl of Fugene-6 reagent was added to 96 μl of KSFM, and the solution was incubated at 25 °C for 5 min. The mixture was then added to 1 μg of involucrin promoter reporter plasmid or, for co-transfection experiments, to 1 μg of involucrin promoter reporter plasmid and 1 μg of kinase expression plasmid. This solution was then incubated at 25 °C for 15 min and then added directly to the cells in 2 ml of KSFM. The final DNA concentration in all groups was adjusted to 2 μg of DNA per 4 μl of Fugene-6 reagent per 10-cm² dish by the addition of empty expression vector. After 24 h, the cells were treated with KSFM in the presence or absence of TPA and/or apigenin. At 48 h post-transfection, the cells were harvested and assayed for luciferase activity. All assays were performed in triplicate, each experiment was repeated a minimum of three times, and luciferase activity was normalized per μg of protein. The transfection efficiency was determined using a green fluorescent protein expression plasmid (42, 43).

**Cell Proliferation**—For cell proliferation assays, keratinocytes were plated at 10,000 cells/cm² in 10-cm² dishes in KSFM and allowed to attach for 24 h. The cells were then treated by the addition of fresh KSFM or KSFM supplemented with 50 ng of TPA/ml in the absence or presence of apigenin. Treatment was continued for 4 days with the addition of fresh medium and TPA/apigenin every 2 days. The cells were then harvested with Hanks’ balanced salt solution containing 0.025% trypsin and 1 mM EDTA and counted using a Coulter counter.

**Cell Extracts and Immunoblot Analysis**—Total cell extracts were prepared from cultured human epidermal keratinocytes as reported previously (32, 39). Bradford Bio-Rad protein assay was used to determine protein concentration. Equal amounts of protein were electrophoresed on 8 or 12% denaturing polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked in 5% nonfat dry milk solution and then incubated with a primary antibody, washed, and exposed to a corresponding horseradish peroxidase-conjugated secondary antibody. Secondary antibody binding was visualized using a chemiluminescent detection system.

**Nuclear Extract Preparation and Gel Mobility Shift Assay**—Human epidermal keratinocytes (75% confluent) growing in KSFM were treated without or with 50 ng/ml TPA in the absence or presence of apigenin 20 μM for 24 h. Nuclear
Apigenin regulation of keratinocyte survival—We next performed experiments designed to identify the DNA element(s) responsible for the TPA and apigenin-dependent regulation of hINV promoter activity. Previous studies showed that the regulatory elements are located in the involucrin promoter proximal regulatory region (nucleotides −241/−7) (36) (Fig. 2A). As shown in Fig. 2B, TPA treatment produces a 6-fold increase in pINV-241 and pINV-128; moreover, this response is abolished by treatment with 20 μM apigenin. In contrast, pINV-110 and pINV-41 are not regulated by these agents. This suggests that key TPA and apigenin-responsive regulatory elements reside in the −241/−110 segment, which encodes an AP1 site, AP1-1 (36, 41, 46, 47). Subsequent studies show that elimination of this site, in construct pINV-241 (AP1-1m), results in a loss of regulation by both agents (Fig. 2C). In contrast, mutation of an ets factor binding site (EBS-2), results in a 10-fold increase in overall promoter activity but does not eliminate the TPA and apigenin-associated regulation, suggesting that the response is specific for the AP1-1 site. This EBS-2 site has a general suppressor activity (41).

RESULTS

Apigenin suppresses involucrin gene expression—Involucrin serves as a marker of keratinocyte terminal differentiation (27, 45), and involucrin expression is increased in cultured keratinocytes following treatment with differentiating agents, such as TPA (36, 41). To assess the impact on keratinocyte differentiation, we examined the effect of apigenin treatment on the increase in hINV promoter activity that is observed following TPA treatment. Keratinocytes were transfected with pINV-2473, a plasmid that encodes the full-length hINV upstream regulatory region and transcription start site linked to the luciferase gene (36) and then treated with TPA in the absence or presence of apigenin. As shown in Fig. 1A, TPA treatment causes an 8-fold increase in pINV-2473 activity, and apigenin produces a concentration-dependent reduction in the TPA-dependent increase. To assess the physiologic relevance of this regulation, we monitored the effects of apigenin on endogenous involucrin expression. Fig. 1B shows that TPA treatment causes an increase in endogenous hINV protein level and that this increase is inhibited by co-treatment with 20 μM apigenin. Fig. 1C summarizes the results from four separate experiments. hINV protein level increases 3.9-fold in response to TPA treatment, and the increase is consistently inhibited by apigenin treatment. To assess whether apigenin influences the level of other keratinocyte differentiation markers, we monitored the impact of TPA and apigenin treatment on type I transglutaminase and filaggrin expression (24). As shown in Fig. 1D, apigenin inhibits the TPA-dependent increase in level of these differentiation markers.

Apigenin regulation of keratinocyte survival—Involucrin promoter activity—We next performed experiments designed to identify the DNA element(s) responsible for the TPA and apigenin-dependent regulation of hINV promoter activity. Previous studies showed that the regulatory elements are located in the involucrin promoter proximal regulatory region (nucleotides −241/−7) (36) (Fig. 2A). As shown in Fig. 2B, TPA treatment produces a 6-fold increase in pINV-241 and pINV-128; moreover, this response is abolished by treatment with 20 μM apigenin. In contrast, pINV-110 and pINV-41 are not regulated by these agents. This suggests that key TPA and apigenin-responsive regulatory elements reside in the −241/−110 segment, which encodes an AP1 site, AP1-1 (36, 41, 46, 47). Subsequent studies show that elimination of this site, in construct pINV-241 (AP1-1m), results in a loss of regulation by both agents (Fig. 2C). In contrast, mutation of an ets factor binding site (EBS-2), results in a 10-fold increase in overall promoter activity but does not eliminate the TPA and apigenin-associated regulation, suggesting that the response is specific for the AP1-1 site. The EBS-2 site has a general suppressor activity (41).
Apigenin Inhibits Signaling Kinase-dependent hINV Promoter Activity—Previous studies show that an nPKC, Ras, MEKK1, MEK3 cascade regulates AP1 factor level and binding to the hINV promoter AP1-1 site (41, 47, 48). It is therefore possible that apigenin may interfere at specific steps in this cascade. In fact, apigenin has been reported to interfere with PKC function by competing with adenosine triphosphate (49–51) and has also been shown to influence MAPK signaling in other systems (52–54). To assess these possibilities, we examined the ability of apigenin to inhibit promoter activity induced by overexpression of PKCδ, -ε, and -η, constitutively active Ras, and MEKK1. It has previously been established that expression of these kinases stimulates hINV promoter activity (31, 47, 48). As shown in Fig. 3A, the novel PKC isoforms, PKCδ, -ε, and -η, increase human involucrin promoter activity when compared with empty vector. Moreover, treatment with 20 μM apigenin suppresses nPKC-dependent hINV promoter activation, suggesting a possible impact on PKC function. Fig. 3B shows that apigenin partially inhibits activation in response to constitutively active Ras (caRas) and nearly completely inhibits MEKK1-dependent promoter activation. This is consistent with findings in HeLa cells that apigenin can antagonize Ras action (55). As shown in Fig. 3C, PKCδ is a major novel PKC isoform present in epidermal keratinocytes.

PKCδ has been shown to have important regulatory actions in epidermis (47, 48, 56–58), and in keratinocytes, the phosphorylation level of PKCδ tyrosine 311 is regulated by a variety of agents (56, 59–62). We therefore examined the impact of apigenin treatment on PKCδ-Y311 phosphorylation. PKCδ-Y311 phosphorylation level increases 2-fold within 15 min after TPA treatment and remains elevated for 120 min. This increase is inhibited by co-treatment with apigenin (Fig. 4A). As shown in Fig. 4B, it is interesting that the level of PKCδ-Y311 phosphorylation returns to nonstimulated levels by 24 h post-treatment. However, even at 24 h, the level of PKCδ-Y311 phosphorylation remains reduced in apigenin-treated cultures. To assess whether reduced PKCδ-Y311 phosphorylation results in a reduction in functional response, we compared the ability of PKCδ wild type and PKCδ-Y311F, in which Tyr311 is converted to phenylalanine and thus cannot be phosphorylated, to regulate hINV promoter activity. As shown in Fig. 4C, PKCδ wild type increases promoter activity, and this increase is inhibited by apigenin treatment. In contrast, PKCδ-Y311F has reduced ability to increase hINV promoter activity, and the residual activity is also inhibited by apigenin. The fact that the PKCδ-Y311F has substantially reduced biological activity suggests that inhibition of PKCδ-Y311 phosphorylation by apigenin may be a physiologically important point of regulation.

Apigenin Suppresses the TPA-dependent Increase in AP1 Factor Binding—The above studies suggest that apigenin may impact downstream responses by inhibiting PKCδ phosphorylation. We therefore examined the impact of apigenin treatment on responses downstream of the MAPK cascade. It is known that this cascade targets AP1 factors, which bind to the hINV promoter AP1 sites to increase gene expression (36). We therefore assessed the impact of apigenin treatment on interaction of AP1 factors with the hINV promoter AP1-1 site. An oligonucleotide encoding the AP1-1 site was labeled with 32P and then incubated with nuclear extract. As shown in Fig. 5A, in the absence of nuclear extract, [32P]AP1-1 is detected as free probe migrating at the gel front (FP). In contrast, in the presence of nuclear extract derived from untreated cells, probe migration is retarded, which is indicative of DNA-protein interaction. The level of protein interaction with [32P]AP1-1 is not altered in nuclear extracts prepared from cells treated with apigenin but is markedly increased in cells treated with TPA. A remarkable finding is that the level of binding is reduced to control levels in extracts derived from cells treated with TPA and apigenin. This finding is consistent with the idea that apigenin influences AP1-1 site occupancy. As shown in Fig. 5B, this binding is AP1 factor-specific, since the binding is reduced by the addition of a 20-fold excess of radioinert AP1-1 but is not reduced by the addition of a 20-fold excess of an oligonucleotide encoding a consensus Sp1 site. To further characterize the interaction, we

**FIGURE 4. Apigenin treatment modulates PKCδ Tyr311 phosphorylation level.** A, keratinocytes were treated with apigenin or TPA for the indicated times, and total extracts were prepared for assay of the PKCδ-Y311 phosphorylation using a PKCδ-phospho-Y311-specific antibody. B, keratinocytes were treated with apigenin or TPA as indicated, and after 24 h total extracts were prepared for assay of the phosphorylation level at tyrosine 311 of PKCδ. Similar results were observed in each of three independent experiments. C, keratinocytes were transfected with 1 μg of pINV-241 in the presence of 1 μg of empty vector or expression vectors encoding PKCδ-EGFP or PKCδ-Y311F-EGFP. At 24 h post-transfection, extracts were prepared for detection of luciferase activity. The results are expressed as mean activity ± S.E., n = 3.
Apigenin Regulation of Keratinocyte Survival

Fra-1 and JunB are part of the DNA-protein complex. Competition with a 20-fold molar excess of AP1-1 is included as an internal control.

These studies suggest that apigenin reduces AP1 factor binding to the AP1-1 site. This could be due to an effect on DNA binding or a reduction in overall AP1 factor level. We suspected the latter explanation, so we monitored the impact of TPA and apigenin treatment on AP1 factor level. Keratinocytes were treated in the presence or absence of TPA and/or apigenin. Nuclear extracts were then prepared, and AP1 factor levels were assayed by immunoblot. As shown in Fig. 6, TPA treatment results in an increase in the level of c-Jun, JunB, JunD, Fra-1, Fra-2, and FosB. Treatment with apigenin suppresses these increases, suggesting that apigenin acts to reduce AP1 factor interaction with DNA by reducing AP1 factor level.

The above experiments suggest that phosphorylation of PKCδ-Y311 is associated with an increase in AP1 transcription factor level, increased binding of AP1 factors to the hINV promoter AP1-1 site, and increased hINV promoter activity. This predicts that PKCδ-Y311 phosphorylation site, should not regulate AP1 factor levels. To assess this, we expressed PKCδ (wild type) or PKCδ-Y311F, which lacks the Tyr311 phosphorylation site, in keratinocytes and monitored the impact on expression of representative AP1 factors. As shown in Fig. 7, expression of PKCδ-Y311 results in increased AP1 factor levels. In contrast, the PKCδ-Y311F mutant does not increase AP1 factor levels. This finding strongly suggests that phosphorylation of PKCδ-Y311 is required for the increase in AP1 factor expression and AP1 factor binding to the hINV promoter AP1-1 site, and the subsequent increase in hINV promoter activity. These findings strongly support the argument that inhibition of PKCδ-Y311 phosphorylation is an important event in the mechanism of apigenin action in keratinocytes.

Proteasome Function—Our previous studies suggest that chemopreventive agents can promote proteasome-dependent degradation of transcription factors (39). We therefore monitored the impact of treatment with the proteasome inhibitor, MG132, on apigenin-dependent degradation of JunB, JunD, and Fra-1. As shown in Fig. 8A, treatment with MG132 reverses the apigenin-dependent reduction in level of these transcription factors, suggesting a role for the proteasome in apigenin-stimulated AP1 transcription factor degradation. It is possible that stabilization of AP1 transcription factors in the presence of MG132 could be due to the parallel stabilization of upstream kinases that control AP1 factor level. However, as shown in Fig. 8B, the level of these kinases is not influenced by treatment with MG132, TPA, or apigenin.

performed gel supershift experiments in which the AP1 factor-DNA complex is exposed to antibodies specific for Fra-1 and JunB. Previous studies show that Fra-1 and JunB interact with the AP1-1 element (36). Interaction of these antibodies results in an additional shift of the gel “supershifted” band. As shown in Fig. 5C, this analysis reveals supershifted bands in extracts incubated with anti-Fra-1 and/or anti-JunB (asterisk). This suggests that

FIGURE 6. Apigenin inhibits the TPA-dependent increase in AP-1 factor level and DNA binding. A, keratinocytes were treated with the indicated levels of apigenin or TPA. After 24 h of treatment, the cells were harvested, and nuclear extracts were prepared for gel mobility shift and supershift analysis. The samples were incubated with [32P]AP1-1 and then electrophoresed on a nondenaturing acrylamide gel. FP, migration of noncomplexed DNA probe ([32P]AP1-1); AP1 (arrow), migration of the gel-shifted complex. B, AP1 factor binding is specific. Nuclear extract prepared from TPA-treated cells was incubated with [32P]AP1-1 in the presence or absence of a 20-fold molar excess of radioinert Sp1c or AP1-1 oligonucleotide (35, 36). The samples were then electrophoresed as outlined above. C, gel mobility supershift analysis. Nuclear extract was prepared from TPA-treated cells and then incubated with [32P]AP1-1 in the presence or absence of a 20-fold molar excess of AP1-1. In parallel lanes, antibodies specific to IgG, Fra-1, or JunB were included in the incubation mixture prior to electrophoresis. The supershifted antibody-transcription factor complexes are indicated by the asterisk.

FIGURE 5. Apigenin inhibits the TPA-dependent increase in AP-1 factor level and DNA binding. A, keratinocytes were treated with TPA at 50 ng/ml in the presence or absence of 20 μM apigenin for 24 h, and nuclear extracts were prepared for assay of AP1 factor levels by immunoblot. Similar results were observed in each of four separate experiments.
Apigenin Regulates Keratinocyte Survival

Apigenin Suppresses Keratinocyte Proliferation—We next monitored the effect of apigenin on keratinocyte proliferation. As shown in Fig. 9A, apigenin causes a concentration-dependent reduction in cell number. Treatment with 10–15 μM apigenin maintains the cell number at a density matching that observed at the start of treatment (control; C). Higher concentrations reduce the cell number below this level. This suggests that apigenin suppresses keratinocyte proliferation. We also monitored the impact of TPA treatment. Administration of 50 ng/ml TPA also results in a suppression of cell number, and cell number is further suppressed by co-treatment with 15 or 20 μM apigenin. Thus, both agents suppress keratinocyte proliferation. As shown in Fig. 9B, treatment with apigenin produces a circular flattened morphology that is also apparent when the cells are co-treated with TPA plus apigenin. This finding suggests that the apigenin phenotype is preferred when cells are treated with both agents. This is similar to the findings in Figs. 5 and 6, which show that apigenin is the dominant controller of AP1 factor level.

Apigenin Treatment Does Not Enhance Apoptosis—The above studies suggest that apigenin suppresses differentiation-associated responses in keratinocytes, which is associated with a reduction in cell number and cell survival. To determine whether this response is associated with apoptosis, we measured the impact of apigenin treatment on mitochondrial integrity, caspase cleavage, and the ratio of Bax to Bcl-xL. Keratinocyte apoptosis involves a shift in the Bcl-xL/Bax ratio, which leads to mitochondrial potential loss followed by procaspase and PARP cleavage and cell destruction (63, 64). We therefore monitored the impact of apigenin on these processes. We first monitored the impact of TPA or apigenin treatment on the Bax to Bcl-xL ratio; an increase in the Bax/Bcl-xL ratio is associated with loss of mitochondrial potential and subsequent apoptosis (63, 64). Treatment with these agents does not alter the Bax/Bcl-xL ratio (Fig. 10A). To confirm the lack of an impact on apoptosis, we monitored the effects of these agents on mitochondrial membrane potential and procaspase and PARP cleavage. Cells were treated with TPA (50 ng/ml) or apigenin (20 μM) for 24 h and then incubated with MitoSensor reagent. Intact mitochondria appear red due to mitochondrial sequestration of the MitoSensor dye, whereas compromised mitochondria fluoresce green (56). As shown in Fig. 10B, neither TPA nor apigenin promote loss of mitochondrial membrane potential. We next monitored the impact on procaspase and PARP cleavage that occurs as a result of cytochrome c release and enhanced apoptosome function following mitochondrial lysis (65). As shown in Fig. 10C, although the levels vary slightly, apigenin treatment does not enhance procaspase or PARP cleavage. As a final method of assessing the apoptotic status of the apigenin- and TPA-treated cells, we monitored the number of sub-G₁ events by cell cycle analysis. As anticipated, there is minimal difference in the number of sub-G₁ cells present following treatment with TPA, apigenin, or the combination, further confirming the lack of apoptosis (Fig. 11).

Apigenin and Green Tea Polyphenol Have Opposing Actions—We have previously demonstrated that EGCG, a chemopreventive antioxidant polyphenol derived from green tea, increases involucrin promoter activity via a nPKC, Ras, MEKK1, MEK3 pathway (32). We therefore examined whether apigenin can oppose this action of EGCG. Keratinocytes were transfected...
Apigenin Suppression the TPA-dependent Activation of hINV Gene Expression—Apigenin is an important nonmutagenic dietary flavonoid derived from vegetables and fruits (4–7). Apigenin has a range of actions and has been shown to have an anticancer role. In epidermis, apigenin protects skin cells from ultraviolet light and chemical carcinogens (14–17). However, the effect of apigenin on normal human keratinocyte function is not well studied. To examine the role of apigenin in normal keratinocytes, we monitored the impact of apigenin treatment on involucrin gene expression, as a marker of cell differentiation. Previous studies indicate that involucrin is regulated via a MEKK1, MEK3, p38 kinase pathway (33, 41, 42, 48). TPA is a protein kinase C-activating agent that promotes keratinocyte differentiation and activation of hINV gene expression via activation of the cascade described above (36). In the present study, we show that apigenin can antagonize this TPA-dependent induction of gene expression.

A range of cell culture and transgenic animal studies implicate protein kinase C isoforms as having a role in the regulation of differentiation and carcinogenesis in epidermis (57, 66–72). In particular, the novel PKC isoforms, PKCδ, -ε, and -η are involved in regulation of involucrin gene expression, and overexpression of these kinases increases involucrin promoter activity (48, 62). We show that apigenin nearly completely inhibits the nPKC-dependent increase in promoter activity. To explore the mechanism, we monitored the impact of apigenin treatment on phosphorylation of PKCδ-Y311. PKCδ-Y311 phosphorylation is thought to have a role in regulating keratinocyte differentiation. In normal human keratinocytes, H2O2 treatment results in increased PKCδ-Y311 phosphorylation that is associated with increased p38δ activity and decreased ERK1/2 activity, and subsequent apoptosis (56). The present studies indicate that PKCδ-Y311 phosphorylation is minimal in resting keratinocytes, is increased at 15–120 min following TPA treatment and returns to control levels by 24 h. This indicates that PKCδ-Y311 phosphorylation is positively associated with TPA treatment and increased expression of AP1 transcription factors and hINV promoter activity. In contrast, phosphorylation of PKCδ-Y311 is markedly reduced by treatment with apigenin, a reduction that is associated with reduced expression of AP1 factors and reduced hINV promoter activity. This suggests that the PKCδ-dependent regulation of downstream responses requires phosphorylation at Tyr311 and that this step is inhibited by apigenin. This conclusion is supported by data showing that the PKCδ tyrosine 311 mutant, PKCδ-Y311F, which cannot be phosphorylated at Tyr311, has a reduced ability to regulate AP1 factor levels and hINV promoter activity.

It should be noted that apigenin treatment also inhibits the hINV promoter activation that is observed following vector-mediated expression of caRas and MEKK1 (41). This finding suggests that the mechanism of apigenin action is more complex than the simple inhibition of PKCδ function and that apigenin suppresses keratinocyte proliferation. A, human keratinocytes (10,000 cells/cm²) were seeded in 35-mm dishes and permitted to attach overnight. The cultures were then treated with the indicated concentration of apigenin for 4 days (left). Normal keratinocytes were plated as above and treated with 50 ng of TPA/ml in the presence of increasing levels of apigenin (right). After 4 days of treatment, the cells were harvested and counted. The open bars (C) indicate the number of cells present on day 0 when treatment was initiated. The values indicate the mean ± S.E., n = 3. B, apigenin influences keratinocyte morphology. Keratinocytes were treated for 24 h with 50 ng/ml TPA, 20 μM apigenin, or a combination. The cells were then photographed.
Apigenin Regulation of Keratinocyte Survival

**A**

| Control | Apigenin (20 μM) | TPA (50 ng/ml) |
|---------|------------------|----------------|
| 30      | +                | +              |
| 30      | +                | +              |
| 45      | β-actin          | β-actin        |

**B**

| Control | Apigenin (20 μM) | TPA (50 ng/ml) | Apigenin + TPA |
|---------|------------------|----------------|---------------|
| 97      | PARP             | caspase-8      | PARP          |
| 45      | procaspase-3     | procaspase-9   | procaspase-8  |
| 30      |                  |                |               |

**C**

| Control | Apigenin (20 μM) | TPA (50 ng/ml) |
|---------|------------------|----------------|
| 97      | β-actin          | β-actin        |

FIGURE 10. **Apigenin does not stimulate caspase-associated apoptosis.**

A, apigenin does not alter the Bax/Bcl-xL ratio. Keratinocytes were treated with the indicated concentration of TPA or apigenin for 24 h prior to preparation of total cell extracts and immunoblot with anti-Bax or anti-Bcl-xL.

B, keratinocytes were treated as above and then incubated with MitoSensor dye. The color and distribution of the dye was then monitored by epifluorescence microscopy.

C, keratinocytes were treated as outlined above. Total extracts were then prepared and assayed for the presence of the indicated proteins. Similar results were observed in each of three independent experiments.

Apigenin is also likely to act at steps downstream of PKCδ-Y311 phosphorylation.

Apigenin Suppresses AP1 Factor Levels; Requirement for Proteasome Activity—AP1 transcription factors are known to have an important role in the epidermis (73–75). Involutrin expression requires AP1 factors binding at AP1 sites in the hINV gene upstream regulatory region (35, 36). This is confirmed in the present studies, which show that mutation of the AP1-1 site, in the context of the pINV-241 reporter plasmid, eliminates the regulation by TPA and apigenin. Previous studies suggest that JunB, JunD, and Fra-1 interact at the AP1-1 site (36). The present study reveals that the TPA-dependent increase in complex formation at the AP1-1 site is reduced to basal levels in the presence of apigenin. It is interesting that apigenin treatment reduces the level of all of the AP1 factors tested, including c-Jun, JunB, JunD, Fra-1, Fra-2, and FosB, and not just those required for regulation of involucrin function. A particularly interesting finding is the observation that the proteasome inhibitor, MG132, inhibits the apigenin-dependent reduction in AP1 factor levels. Immunoblot analysis shows that this is a specific effect on AP1 factor level that is not due to MG132 regulation of level of the upstream signaling kinases that normally control AP1 factor level.

At present we believe that the ability of proteasome inhibitors to reverse the apigenin-dependent reduction in AP1 factor level is a general effect that is independent of the mechanism whereby apigenin inhibits hINV gene expression. However, additional studies will be necessary to confirm this hypothesis, since chemopreventive agents have been proposed to interface with the proteasome system. In fact, some studies suggest that proteasome function is required for chemoprevention. For example, treatment with a 26 S proteasome inhibitor, lactacystin, interferes with curcumin-dependent degradation of cyclin D1 in breast and prostate cancer cells (76). In another study, apigenin stimulates degradation of HER2/neu in breast cancer cells, and this action is reduced by treatment with proteasome inhibitor (77). In contrast, apigenin has also been proposed to directly modify proteasome activity. In Jurkat T leukemia cells, for example, apigenin treatment is associated with accumulation of ubiquitinated forms of Bax, increased caspase-3 and poly(ADP-ribose) polymerase activity, leading to apoptosis (78). This is attributed to an apigenin-dependent inhibition of proteasome function. Thus, the mechanism of apigenin action is likely to be complex and may vary depending upon the cell type and end point. In keratinocytes, treatment with the chemopreventive agent, curcumin, inhibits hINV promoter activity via a mechanism that involves a reduction in the level of the C/EBP transcription factor (39). The reduction in C/EBP level is reversed by treatment with the proteasome inhibitor, MG132, suggesting that the proteasome function is required for curcumin action (39). These findings suggest an interesting parallel in that two chemopreventive agents, each of which acts by a different general mechanism, both ultimately act to reduce transcription factor levels via a mechanism that requires proteasome function.

**Apigenin and TPA; Common and Opposing Actions**—TPA is known to enhance keratinocyte differentiation, which is evidenced by the TPA-dependent increase in involucrin promoter activity (36). In contrast, TPA does not enhance apoptosis, which is evidenced by the absence of effect of TPA treatment on mitochondrial stability or caspase cleavage (see Fig. 8). An interesting feature of the opposing action of TPA and apigenin is that apigenin is dominant. Thus, apigenin treatment inhibits the TPA-dependent increase in hINV gene expression and also produces the dominant morphological change. This suggests that apigenin is inhibiting TPA action at an early step in the signaling cascade. Such a supposition is consistent with our evidence that apigenin treatment reduces PKCδ-Y311 phosphorylation and that apigenin partially inhibits the promoter activating action of constitutively active Ras and MEKK1. However, TPA and apigenin also share common actions. For example, both inhibit cell proliferation, and combined treatment with both agents results in enhanced suppression of proliferation. Thus, the interplay between these agents is likely to be complicated and is likely to involve multiple signaling pathways.
Chemopreventive Agents and Regulation of Keratinocyte Function—It is interesting that apigenin antagonizes the action of another anti-cancer chemopreventive agent, EGCG. EGCG is the major bioactive polyphenol present in green tea and is an important antioxidant inhibitor of tumor cell proliferation and tumor formation (79, 80). EGCG also has important functions in normal keratinocytes, since our recent studies show that it acts to inhibit cell proliferation and enhance differentiation (32, 39, 81–83) but does not stimulate apoptosis (81, 82). EGCG treatment increases involucrin gene expression via the same pPKC, Ras, MEKK1, MEK3, p38/ERK1/2 cascade that is activated by TPA (32). Our present studies show that apigenin inhibits EGCG-dependent involucrin gene expression. Taken together, our studies argue that chemopreventive agents have very different mechanisms of action in normal keratinocytes. EGCG, for example, suppresses cell proliferation and enhances differentiation but does not regulate apoptosis (32, 39, 81); curcumin inhibits proliferation and enhances apoptosis but does not promote differentiation (39); and apigenin suppresses cell differentiation and proliferation but does not enhance apoptosis. These findings illustrate that different chemopreventive agents can produce very different keratinocyte responses and suggest that it will be important to consider these features when designing chemoprevention strategies that utilize more than one chemopreventive agent.

REFERENCES
1. Hertog, M. G., Feskens, E. J., Hollman, P. C., Katan, M. B., and Kromhout, D. (1994) Nutr. Cancer 22, 175–184
2. Hertog, M. G., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovic, S., Pekkanen, M., Simic, B., Toshima, H., Feskens, E., Hollman, P., and Katan, M. (1995) Arch. Intern. Med. 155, 381–386
3. Hollman, P. C., Feskens, E. J., and Katan, M. B. (1999) Proc. Soc. Exp. Biol. Med. 220, 198–202
4. Matern, U., Heller, W., and Himmelspach, K. (1983) Eur. J Biochem. 133, 439–448
5. Ross, J. A., and Kasum, C. M. (2002) Annu. Rev. Nutr. 22, 19–34
6. Peryt, B., Szymczyk, T., and Lesca, P. (1992) Mutat. Res. 269, 201–215
7. Engelhardt, U. H., Finger, A., and Kuhr, S. (1993) Z. Lebensm. Unters. Forsch. 197, 239–244
8. Lepley, D. M., Li, B., Birt, D. F., and Pelling, J. C. (1996) Carcinogenesis 17, 2367–2375
9. Gupta, S., Afaq, F., and Mukhtar, H. (2002) Oncogene 21, 3727–3738
10. Wang, W., Heideman, L., Chung, C. S., Pelling, J. C., Koehler, K. J., and Birt, D. F. (2000) Mol. Carcinog. 28, 102–110
11. Yin, F., Giuliano, A. E., and Van Herle, A. J. (2001) Anticancer Res. 21, 413–420
12. Chaumontet, C., Bex, V., Gaillard-Sanchez, L., Seillan-Heberden, C., Suschetet, M., and Martel, P. (1994) Carcinogenesis 15, 2325–2330
13. Fotsis, T., Pepper, M. S., Aktas, E., Breit, S., Rasku, S., Adlercreutz, H., Wahala, K., Montesano, R., and Schweigerer, L. (1997) Cancer Res. 57, 2916–2921
