Antioxidants are important candidate agents for the prevention of disease. However, the possibility that different antioxidants may produce opposing effects in tissues has not been adequately explored. We have reported previously that (−)-epigallocatechin-3-gallate (EGCG), a green tea polyphenol antioxidant, stimulates expression of the keratinocyte differentiation marker, involucrin (hINV), via a Ras, MEKK1, MEK3, p38α signaling cascade (Balasubramanian, S., Efimova, T., and Eckert, R. L. (2002) J. Biol. Chem. 277, 1828–1836). We now show that EGCG activation of this pathway results in increased CCAAT/enhancer-binding protein (C/EBP) factor level and increased complex formation at the hINV promoter C/EBP DNA binding site. This binding is associated with increased promoter activity. Mutation of the hINV promoter C/EBP binding site eliminates the regulation as does expression of GADD153, a dominant-negative C/EBP factor. In contrast, a second antioxidant, curcumin, inhibits the EGCG-dependent promoter activation. This is associated with inhibition of the EGCG-dependent increase in C/EBP factor level and C/EBP factor binding to the hINV promoter. Curcumin also inhibits the EGCG-dependent increase in endogenous hINV levels. The curcumin-dependent suppression of C/EBP factor level is inhibited by treatment with the proteasome inhibitor MG132, suggesting that the proteasome function is required for curcumin action. We conclude that curcumin and EGCG produce opposing effects on involucrin gene expression via regulation of C/EBP factor function. The observation that two antioxidants can produce opposite effects is an important consideration in the context of therapeutic antioxidant use.

The human epidermis is a stratifying squamous epithelium comprised of a single, basally located layer of proliferating keratinocytes and multiple suprabasal layers of differentiating keratinocytes (1–4). During differentiation, keratinocytes undergo a choreographed series of morphological and biochemical changes that result in the assembly of a protective cornified envelope (5). Involucrin is a component of this structure that is specifically expressed in the epidermal suprabasal layers (6, 7). Activation of involucrin gene expression is controlled by a p38 MAPK signaling cascade. This cascade includes novel protein kinase C, Ras, MEKK1, MEK3, and p38α (8), and it targets various transcription factors (9–11). These factors, in turn, bind to the hINV promoter upstream regulatory region (nucleotides −2473 to −1) to activate hINV gene expression (9, 12). In the present manuscript, we utilize this system to study the role of antioxidants in regulating keratinocyte function.

Antioxidants comprise a collection of agents, derived from various sources, that are potential disease-preventive agents (13–15). We have reported recently that (−)-epigallocatechin-3-gallate (EGCG), a green tea polyphenol antioxidant, increases hINV promoter activity and endogenous hINV expression in normal epidermal keratinocytes (8). An important question is whether individual antioxidants produce similar or opposing effects in cells. This is important because these agents are being considered for use in treating a host of diseases, and the possibility exists that simultaneous treatment with structurally dissimilar antioxidants may produce opposing effects. To investigate this possibility, we have examined the interplay between EGCG and a second antioxidant, curcumin, in regulating involucrin gene expression.

EGCG is the major bioactive polyphenol component of green tea and has been reported to regulate a wide range of processes in a variety of cell types (16–18). Curcumin (diferuloylmethane), commonly called turmeric, is a polyphenol derived from the plant Curcuma longa (19–21). Both agents are effective cancer therapeutic and prevention agents. However, because of the varying chemical structure of these agents, some of their effects may be unrelated to their antioxidant properties. Thus, it is important to evaluate the effects of individual antioxidants to determine whether they produce contributing or opposing effects. In the present study, we show that curcumin opposes the EGCG-dependent activation of involucrin gene expression by inhibiting the EGCG-dependent increase in C/EBP transcription factor levels. These finding indicate that not all antioxidants produce parallel changes in human keratinocyte function and indicate that potential opposing effects of these agents must be considered when antioxidants are proposed for therapeutic use.
At 24 h after transfection, the cells were treated for 24 h in the presence or absence of 40 \( \mu \)M EGCG, which encodes an intact promoter. In plasmids pINV-241(EBS-2m) and pINV-241(C/EBPm), the EBS-2 or the C/EBP site, respectively, is mutated (9). pINV-241, has been described previously (11). To create pINV-241(C/EBPm), the C/EBP site mutant, the fragment containing the wild type C/EBP site (5'-GCTGCTTAAG-3'), was released as part of a larger fragment by digestion of pINV-241 with ApaI/PstI and replaced with the identical segment containing a mutated C/EBP site (5'-GCTGCTTAAG-3'), which was isolated from pEECMV, was provided by Dr. Dennis Templeton (27).

FIG. 1. A, structure of hINV promoter-luciferase reporter plasmids. The luciferase gene is indicated by the filled rectangle, and the start site and direction of transcription are indicated by the arrow. The nucleotide position is given relative to the transcription start site, and the C/EBP, activator protein-1 (AP1), and ets factor binding sites are indicated. pINV-241(EBS-2m) and pINV-241(C/EBPm) have mutations at the EBS-2 or the C/EBP site, respectively. B, EGCG regulation of hINV promoter activity requires intact C/EBP factor DNA binding site. Human keratinocytes were grown in KSFM until 70% confluent and then transfected with the indicated hINV promoter-luciferase reporter constructs. Plasmid pINV-241 encodes an intact promoter. In plasmids pINV-241(EBS-2m) and pINV-241(C/EBPm), the EBS-2 or the C/EBP site, respectively, is mutated (9). At 24 h after transfection, the cells were treated for 24 h in the presence or absence of 40 \( \mu \)M EGCG. The cells were then harvested for extraction and measurement of luciferase activity (28). The errors bars represent the S.E. This experiment was repeated three times with similar results.

C, EGCG regulation of endogenous hINV level. Keratinocytes were treated for 48 h with 40 \( \mu \)M EGCG. Extracts were prepared and electrophoresed for detection of hINV protein with anti-hINV. \( \beta \)-Actin was detected as a control to normalize loading. The gels were scanned, and the results were plotted as hINV level (arbitrary units) after normalizing to the \( \beta \)-actin level.

MATERIALS AND METHODS

Chemicals and Reagents—Epigallocatechin-3-gallate and curcumin were purchased from Sigma. EGCG was prepared as 1000-fold stock in sterile distilled water. MG132 (Z-Leu-Leu-Leu-CHO) was purchased from Biomol. Keratinocyte serum-free medium (KSFM), gentamicin, trypsin, and Hanks’ balanced salt solution were obtained from Invitrogen. Dispase was from Roche Applied Science. The pGL2-Basic plasmid and chemiluminescent luciferase assay system were purchased from Promega. Chemiluminescence was measured using a Berthold luminometer, and synthetic oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer. \( \gamma ^{32} \)P-ATP was purchased from PerkinElmer Life Sciences. C/EBP transactivation factor-selective rabbit polyclonal antibodies specifying C/EBP\( _{a} \) (sc-61X, 1:500), C/EBP\( _{b} \) (sc-636X, 1:500), C/EBP\( _{\beta} \) (sc-638X, 1:500), and GADD153 (sc-783, 1:500) were obtained from Santa Cruz Biotechnology. An antibody specific for \( \beta \)-actin from Sigma (A5441) was used for immunoblot at a dilution of 1:10,000.

Plasmids—The structure of the hINV promoter reporter plasmid, pINV-241, has been described previously (11). To create pINV-241(C/EBP\( _{a} \)), the C/EBP site mutant, the fragment containing the wild type C/EBP site (5'-GCTGCTTAAG-3'), was released as part of a larger fragment by digestion of pINV-241 with ApaI/PstI and replaced with the identical segment containing a mutated C/EBP site (5'-GCTGCTTAAG-3') (9). The modified nucleotides are underlined. C/EBP\( _{a} \) was kindly provided by Dr. David Samols (Case Western Reserve University School of Medicine) (22). C/EBP\( _{b} \) (CRP2, rat), and C/EBP\( _{\beta} \) (CRP3, mouse) were expressed using pMEX and provided by Dr. Peter Johnson (23). GADD153 and pCMV-neo were provided by Dr. Nikki Holbrook (24, 25). Constitutively active Ras (Ras\( _{G_{12}s} \)) was obtained from Dr. Michael Simonson (26). Wild type MEKK1, cloned in pEECMV, was provided by Dr. Dennis Templeton (27).

Cell Transfection and Luciferase Assay—Normal human foreskin keratinocytes were isolated and cultured as described previously (9). Keratinocytes (60% confluent, third passage) were transfected in 9.5-cm\(^2\) area dishes. FuGENE 6 transfection reagent was added to KSFM at a final concentration of 4% and incubated at 25 °C for 5 min. The mixture was then added to 2 \( \mu \)g of involucrin promoter reporter plasmid or, for co-transfection experiments, with 1 \( \mu \)g of involucrin reporter plasmid and 1 \( \mu \)g of a second plasmid. The mixture was incubated at 25 °C for 15 min and then added directly to the cultures in 2 ml of KSFM. The final DNA concentration in all groups was adjusted to 2 \( \mu \)g of DNA/4 \( \mu \)l of FuGENE 6 reagent/9.5-cm\(^2\) dish area by addition of empty expression vector. After 24 h, the cells were incubated with RSFM in the presence or absence of EGCG and/or curcumin. After an additional 24 h, the cells were washed with phosphate-buffered saline, dissolved in 200 \( \mu \)l of cell culture lysis reagent (Promega), and harvested by scraping. Luciferase activity was assayed immediately using Promega luciferase assay kit and a Berthold luminometer. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. Luciferase activity was normalized per \( \mu \)g of protein. Transfection efficiency was normalized using a green fluorescent protein-encoding expression vector.

Preparation of Nuclear Extracts and Gel Mobility Shift Analysis—Cultured keratinocytes (80% confluent) were treated for 24 h with KSFM or with KSFM containing 40 \( \mu \)M EGCG and/or 20 \( \mu \)M curcumin prior to preparation of nuclear extracts (8). The protein content was determined using Bradford reagent (Bio-Rad). Binding of transcription factors to the hINV promoter C/EBP site was detected using gel mobility shift assay (8). Five \( \mu \)g of nuclear extract was incubated for 10 min at room temperature in a total volume of 20 \( \mu \)l containing 40 mM HEPES (pH 7.6), 10% glycerol, 200 mM KC1, 10 mM dithiothreitol, 2
μg/ml poly(dI-dC), and 100,000 cpm of radioactive double-stranded DNA oligonucleotide. The oligonucleotide, 5′-GCTTGGCTGGTAA-GATGCGT-3′, that encodes the hINV C/EBP binding site (C/EBP site is underlined) was end-labeled using polynucleotide kinase and [γ-32P]ATP. For competition studies, radiolabeled DNA competitor was added to the DNA binding reaction at 10- or 100-fold molar excess. Protein-DNA complexes were resolved on a 6% non-denaturing polyacrylamide gel using 0.5× Tris/borate/EDTA running buffer. The gel was dried, and the radioactivity was detected by autoradiography.

Immunoblot Analysis—Total cell extracts were prepared from cultured human keratinocytes as described previously (8). Equivalent amounts of protein were electrophoresed on 10% denaturing polyacrylamide gels and transferred to nitrocellulose. The membranes were blocked, incubated with a specific primary antibody, washed, and exposed to an appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection (Amersham Biosciences) was used to visualize secondary antibody binding.

RESULTS

C/EBP Site Is Required for EGCG-dependent hINV Promoter Regulation—We first examined the effects of EGCG treatment on hINV promoter activity. Fig. 1A shows a schematic of the hINV promoter constructs. Promoter activity assays, shown in Fig. 1B, indicate that EGCG treatment produces a 4-fold increase in activity of the intact hINV promoter construct, pINV-241. Mutation of the C/EBP site, pINV-241(C/EBPm), results in a loss of the EGCG-dependent regulation. As a control, we examined the effects of EGCG treatment on a promoter construct in which an ets factor binding site (EBS), pINV-241(EBS-2m), is mutated. This mutation results in an overall increase in basal and EGCG-stimulated promoter activity, but the 4-fold EGCG-dependent increase is still observed. These results suggest that the C/EBP site is specifically required for EGCG-dependent gene expression. Fig. 1C confirms relevance of the response by showing that endogenous hINV gene expression is increased by 4-fold as measured by increased presence of hINV protein. Additional studies reveal a parallel increase in hINV mRNA (not shown).

EGCG Increases C/EBPα-dependent hINV Promoter Activation—The above results suggest that C/EBP factors may be important mediators of the EGCG-dependent response. To test this further, we evaluated whether C/EBP factors influence hINV promoter activity. Cells were transfected with pINV-241 in the presence of expression vectors encoding C/EBPα, -β, or -δ. Fig. 2A shows that hINV promoter activity is markedly increased in the presence of C/EBPα, with a lesser increase observed in the presence of C/EBPβ and -δ. In contrast, GADD153, a dominant-negative C/EBP family member that suppresses function of other C/EBP proteins by inhibiting their interaction with DNA (9), completely inhibits the EGCG-dependent increase (Fig. 2B). Basal activity is also inhibited, indicating that basal transcription also relies on C/EBP factors. Fig. 2C confirms that C/EBPα, C/EBPβ, C/EBPδ, and GADD153 are each expressed when cells are treated with the corresponding expression vector. Longer exposure of these films revealed the presence of endogenous C/EBPα and C/EBPδ; however, C/EBPβ was not detected (not shown).

The above results suggest that EGCG may control hINV promoter activity by regulating C/EBP factor level. To determine whether EGCG regulates endogenous C/EBP factor levels, nuclear extracts were prepared from keratinocytes after treatment for 24 h with 40 μM EGCG. C/EBP factor levels were then assayed by immunoblot. As shown in Fig. 3A, EGCG treatment substantially increases C/EBPα and C/EBPβ level. C/EBPδ could not be detected. To determine whether the increased C/EBP level correlates with increased C/EBP binding to the hINV promoter C/EBP DNA element, we performed gel mobility shift studies. Cells were treated with vehicle or 40 μM EGCG. After 24 h the cells were harvested, and nuclear extracts were incubated with 32P-labeled C/EBP oligonucleotide (32P-C/EBP). As shown in Fig. 3B, basal protein binding to the hINV C/EBP element is markedly increased following EGCG treatment. Moreover, addition of radiolabeled competitor oligonucleotide causes a reduction in DNA binding, suggesting that the binding is specific. In addition, this binding is not competed by an unrelated oligonucleotide, Sp1c, that encodes a consensus Sp1 site (11).

Curcumin Antagonizes the EGCG-dependent Regulation—The above studies indicate that EGCG increases hINV gene expression, both the endogenous gene and the promoter, and suggest that this increase is dependent upon a functional C/EBP site and also elevated C/EBP levels. We next assessed the role of another antioxidant, curcumin. As shown in Fig. 4, unlike EGCG, curcumin treatment does not increase hINV promoter activity. In addition, incubation of curcumin with EGCG results in inhibition of the EGCG-dependent increase in promoter activity. To assess the concentration dependence of this response, cells were treated with or without an optimal concentration of EGCG (40 μM) and challenged with increasing concentrations of curcumin. As shown in Fig. 5A, curcumin suppresses the EGCG-dependent increase in promoter activity.
promoter activation. This suggests that the site of curcumin with curcumin inhibits both caRas- and MEKK1-dependent activity is associated with reduced binding at the hINV promoter C/EBP site.

**Proteasome Inhibition Reverses Curcumin-dependent Reduction in C/EBP Factor Level**—We next investigated the mechanism of this regulation. Several studies suggest that C/EBP factor level may be regulated by proteasome-dependent mechanisms (29, 30). To assess the impact of proteasome function on the curcumin-dependent reduction of C/EBPα and -β levels, cells were incubated with curcumin in the absence or presence of the proteasome inhibitor MG132. As shown in Fig. 8, treatment with curcumin results in a reduction in endogenous C/EBPα and C/EBPβ levels. This reduction is reversed by treatment with the proteasome inhibitor MG132.

We next assessed the effect of C/EBPα overexpression and proteasome activity on hINV promoter activity. As shown in the upper panel in Fig. 9A, C/EBPα overexpression increases hINV promoter activity, and this increase is inhibited by curcumin treatment. The lower panel in Fig. 9A shows that the curcumin-dependent reduction in promoter activity is associated with a parallel reduction in C/EBPα levels. The upper panel in Fig. 9B shows that the curcumin-dependent reduction in hINV promoter activity is reversed by addition of MG132, and the lower panel confirms that the MG132-dependent restoration of promoter activity is associated with restored C/EBPα levels. The stable level of β-actin in these extracts suggests that the curcumin-dependent reduction in C/EBP factor level is not due to a generalized nonspecific reduction in protein expression.

**DISCUSSION**

Antioxidants are important agents that are currently being tested for treatment or prevention of a host of diseases including cancer (31) and various skin diseases (32–35). For this reason, it is important to identify their mechanism of action. Most studies of antioxidant-dependent chemopreventive action
examine the effects of a single antioxidant. However, in vivo, cells are simultaneously exposed to a variety of antioxidants. This fact argues that it is important to evaluate the effects of simultaneous presence of these agents. In the present study, we compare the effects of EGCG and curcumin on keratinocyte function. We have used regulation of involucrin gene expression as an end point. Involucrin is a marker of keratinocyte differentiation that is selectively expressed as cells differentiate (36, 37). A recent study identified an EGCG-activated regulatory pathway in normal human keratinocytes that includes Ras, MEKK1, MEK3, and p38 and showed that activation of this pathway results in increased involucrin gene expression (8). In the present study, we utilized this system to gain insights regarding the effects of co-treatment with EGCG and curcumin, two antioxidants, on keratinocyte gene expression.

Our study shows that C/EBP transcription factors are downstream targets of EGCG-associated activation of the p38 MAPK cascade. Treatment of keratinocytes with EGCG increases endogenous C/EBP factor levels and complex formation on the hINV promoter C/EBP response element. The increase in C/EBP factor level appears to be important for transcriptional activation, as delivery of exogenous C/EBP also enhances the level of involucrin transcription. GADD153, which functions as a dominant-negative C/EBP factor, inhibits the EGCG-dependent increase in promoter activity by forming inactive complexes with other C/EBP factors (24, 25). CCAAT/enhancer-binding proteins comprise a family of basic region/leucine zipper transcription factors that regulate normal cellular differentiation in a variety of tissues (23, 38, 39). A role for C/EBP factors in regulating involucrin gene expression is not unanticipated, as C/EBP factors are expressed in a differentiation-dependent manner in the skin and play a role in regulating epidermal differentiation (40, 40–42). C/EBP factors also have a role in mediating the response of the hINV promoter to phorbol ester (9). Although EGCG has been reported to regulate function of several tran-

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**Fig. 5.** Curcumin inhibition is concentration-dependent. A, human keratinocytes were grown in KSFM until 70% confluent and then transfected with the pINV-241 luciferase reporter construct (9). At 24 h after transfection, the cells were treated for 24 h in the presence or absence of 40 μM EGCG in the presence of increasing curcumin concentration. Promoter activity was monitored by assaying luciferase activity (28). The error bars represent the S.E. (n = 3). B, curcumin inhibits the EGCG-dependent increase in endogenous hINV protein levels. Cells were treated with the indicated concentration of EGCG or curcumin for 24 h, and extracts were prepared to monitor hINV and β-actin levels by immunoblot. The -fold change was determined by laser densitometric scanning after normalizing to the β-actin signal. This experiment is representative of three separate experiments.

**Fig. 6.** Curcumin inhibits caRas- and MEKK1-dependent hINV promoter activity. A, EGCG regulates hINV gene expression via a MAPK cascade. B and C, human keratinocytes were grown in KSFM until 70% confluent and then transfected with the 1 μg of pINV-241 luciferase reporter construct (9) in the presence or absence of 1 μg of empty vector (EV) or caRas or MEKK1 expression vector. At 24 h after transfection, the cells were treated for 24 h in the presence of the indicated concentration of curcumin. The cells were then harvested for extract preparation and measurement of luciferase activity (28). The error bars represent the S.E. (n = 3).
Fig. 7. Curcumin inhibits EGCG-dependent increase in the C/EBP factor level and also C/EBPα binding to the hINV promoter C/EBP site. A, cells were treated with EGCG and curcumin for 24 h, and extracts were prepared to monitor C/EBPα, C/EBPβ, and β-actin levels by immunoblot. The fold-change was determined by laser densitometric scanning after normalizing to the β-actin signal. This experiment is representative of three separate experiments. B, curcumin reduces EGCG-dependent C/EBP factor binding to hINV promoter C/EBP site. Cells were treated for 24 h with the indicated concentration of EGCG or curcumin. Nuclear extracts (NE) were prepared for gel mobility shift analysis. The leftmost lane shows migration of free 32P-C/EBP probe in the absence of nuclear extract. FP and C/EBP indicate migration of the free probe and the C/EBP band, respectively.

Fig. 8. MG132 inhibits the curcumin-dependent reduction in C/EBP factor level. Keratinocytes were incubated in the presence of curcumin and/or MG132 for 24 h prior to preparation of nuclear extracts. Equivalent amounts of nuclear extract were electrophoresed on parallel lanes of a 10% denaturing acrylamide gel and then incubated with anti-C/EBPα or anti-C/EBPβ followed by incubation with an appropriate secondary antibody. Secondary antibody binding was then visualized using chemiluminescent detection reagents.

Curcumin Reduces C/EBP Transcription Factor Levels via a Proteasome-dependent Mechanism—C/EBP factors are the penultimate regulators in the involucrin signaling cascade (9). Direct assay of C/EBP factor levels indicates that curcumin reduces endogenous C/EBPα and β levels and C/EBP factor binding to the hINV promoter C/EBP site. Moreover, curcumin also inhibits the increase in involucrin expression observed following C/EBPα overexpression, a response that is associated with a substantial reduction in C/EBPα levels. These findings suggest that curcumin may regulate C/EBP factor turnover. C/EBP factor level has been reported to be regulated by the proteasome. In BALB/MK2 cells, for example, C/EBPα has a half-life of 1 h, and treatment with proteasome inhibitors increases the half-life and causes a 5-fold increase in C/EBPα level (30). In addition, C/EBPβ and β levels are increased in Caco-2 intestinal epithelial cells following treatment with proteasome inhibitors (29). Moreover, resistance to proteasome-mediated degradation is provided by leucine zipper-dependent C/EBP factor dimer formation (66). In addition, several reports indicate that curcumin modulates proteasome function. Nakayama et al. (67) found that curcumin treatment inhibits the proteasome inhibitor-dependent transcriptional increase in monocyte chemoattractant protein one expression in mesangial cells, and Aggarwal and co-workers (60) showed that the proteasome inhibitor lactacystin inhibits the curcumin-dependent reduction in cyclin D1 level in breast cancer cells.

Our present studies show that treatment with MG132, a proteasome inhibitor, blocks the curcumin-dependent reduction in C/EBP factor level and also inhibits the curcumin-dependent reduction in involucrin promoter activity. These findings suggest that curcumin acts to reduce involucrin gene expression by reducing C/EBP factor levels and that this decrease requires proteasome function. However, additional future studies will be needed, as we cannot rule out the possibility that curcumin may inhibit at multiple points in the MAPK cascade to reduce overall C/EBP factor levels or that curcumin

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may inhibit C/EBP factor transcription.

Curcumin and EGCG Both Reduce Keratinocyte Cell Number—The above results identify opposing effects of EGCG and curcumin on hINV promoter activity, endogenous hINV expression, and C/EBP transcription factor level. A key question is whether these agents have opposing actions with respect to other keratinocyte end points. As noted above, treatment of normal human oral epithelial cells with EGCG or curcumin results in a reduction in cell number (64). Our study indicates that both EGCG and curcumin reduce normal human keratinocyte cell number (not shown). Thus, the response to simultaneous treatment with more than one antioxidant is dependent upon the end point being monitored. Involucrin gene regulation is inversely regulated by these agents, but both agents inhibit proliferation. These findings are important, as they suggest that specific antioxidants differentially regulate gene expression, perhaps in an end point-specific manner. Such considerations may complicate efforts to predict the outcome that can be expected following treatment of skin diseases with antioxidants.

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