CCAAT/Enhancer-binding Proteins

A ROLE IN REGULATION OF HUMAN INVOLUCRIN PROMOTER RESPONSE TO PHORBOL ESTER

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The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent inducer of keratinocyte differentiation and of involucrin gene expression. In the present study we show that a CCAAT/enhancer-binding protein (C/EBP) site in the proximal regulatory region is required for the phorbol ester response. Mutation of the C/EBP site results in the loss of basal and TPA-responsive activity. Gel mobility supershift analysis shows that C/EBPα binding to this site is increased by TPA treatment. Moreover, cotransfection of the human involucrin reporter plasmid with C/EBPα increases promoter activity to an extent comparable with TPA treatment. Mutation of the C/EBP-binding site eliminates these responses. Transfection experiments using GADD153 to create C/EBP-null conditions confirm that C/EBP factors are absolutely required for promoter activity and TPA responsiveness. C/EBPβ and C/EBPδ inhibit both TPA- and C/EBPα-dependent promoter activation, indicating functional differences among C/EBP family members. These results suggest that C/EBP transcription factor activity is necessary for basal promoter activity and TPA response of the involucrin gene.

The cornified envelope is a covalently cross-linked layer of protein that is formed by epidermal keratinocytes during the final stages in differentiation (1, 2). Involucrin (hINV) is a precursor of the keratinocyte cornified envelope that functions as a glutamyldonor and amine acceptor in the transglutaminasedependent cross-linking reaction (1, 3–7). For proper envelope formation the transglutaminase enzyme and its substrates (the envelope precursors) must be expressed at the appropriate time and level during the differentiation process. Abnormal expression or lack of expression can result in disease (8–10).

Involucrin is exclusively expressed in the suprabasal epidermal layers (1, 7, 11–13). The mechanisms that regulate hINV expression during keratinocyte differentiation are an area of active investigation (14–22). The proximal regulatory region (PRR) of the hINV promoter is located between positions –241 and –7 relative to the start of transcription (20, 21). The PRR drives nearly one-half of the activity of the promoter. Site specific mutation experiments indicate that an activator protein 1 (AP1)-binding site, AP1-1, located within the PRR, is absolutely required for promoter activity (20). This site interacts with JunB, JunD, and Fra-1 (20). In addition to the AP1-1 site, this region contains a C/EBP site that is necessary for promoter activity (14).

C/EBP factors comprise a family of related bZIP (basic region leucine zipper) DNA-binding proteins that regulate transcription. This family includes C/EBPα, C/EBPβ, C/EBPδ, GADD153, CHOP, and LAP (23–28). C/EBP factors have been shown to differentially modulate transcription and differentiation in adipocytes, myelomonocytic cells, and ovarian follicles (24, 26, 29, 30). Based on previous studies showing that the C/EBP-binding site of the hINV promoter is important for activity (14), we hypothesized that C/EBP factors may have a role in regulating hINV expression during keratinocyte differentiation. In the present study we show that each C/EBP protein differentially regulates hINV promoter activity via the hINV promoter C/EBP site and that C/EBP factor binding to this site is dramatically increased following treatment of keratinocytes with 12-O-tetradecanoylphorbol-13-acetate (TPA). We show that C/EBPα is a component of this complex. We also use an inhibitory member of the C/EBP family, GADD153, to show that C/EBP factor activity is required for the TPA-dependent increase in activity.

MATERIALS AND METHODS

Reagents—[γ-32P]ATP (3000 Ci/mmol) was purchased from NEN Life Science Products. Keratinocyte serum-free medium (KSFM), trypsin, Hank’s balanced salt solution, gentamicin, and Lipofectin were obtained from Life Technologies, Inc. The pGL2 plasmid and the chemiluminescent luciferase assay systems were obtained from Promega. Phorbol ester (TPA) and dimethyl sulfoxide (Me2SO) were obtained from Sigma. Chemiluminescence was measured using a Berthold lumimeter, and synthetic oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer.

Tissue Culture—Human foreskin keratinocytes were isolated and cultured as described (20, 21). The cells were passaged at a split ratio of 1:5 when 70% confluent and used for transfection at the third passage.

Plasmid Construction—The structure of the hINV promoter reporter plasmid, pINV-241, has been described (20). To create the C/EBP site mutant, the fragment containing the wild type C/EBP site (5'GCTGAGATCT-3') was replaced with a larger fragment by digestion of pINV-241 with ApaI/PstI and replaced with the identical segment containing a mutated C/EBP site (5'-GCTGAGATCT-3'). The modified nucleotides are underlined. The structure of the mutated AP1-1 site in pINV-241(AP1-1m) has been previously described (20). The junction between the hINV gene sequences and the luciferase reporter gene sequence is identical in all constructs.

Cell Transfection and Luciferase Assay—Keratinocytes (60% confluent) were transfected in 60-mm-diameter dishes. Lipofectin reagent (16 μg) and 4.0 μg of test plasmid were added to cells in 3 ml of KSF M and incubated for 5 h at 37 °C. After 5 h, additional KSF M (3 ml) was added, and the incubation was continued for another 19 h. After 24 h in fresh
KSFM, cells were washed and treated for 24 h with KSFM or KSFM containing 50 ng/ml TPA (delivered from a 5 mg/ml stock in Me2SO) (20, 21). Control groups received the Me2SO vehicle. For cotransfection experiments, involucrin reporter plasmid (2.5 μg) was transfected with various concentrations of transcription factor expression plasmid. The final transfection solution concentration was maintained constant by addition of an empty expression vector. The C/EBP factors C/EBPα (CRP2, rat) and C/EBPβ (CRP3, mouse) were expressed using pMEX (Dr. Peter Johnson of the Frederick Cancer Research Center) (25). C/EBPα was obtained from Dr. David Samols (Case Western Reserve University) (31). GADD153 and pCMV-neo were obtained from Dr. Nikki Holbrook (27, 28). The cells were harvested and assayed for luciferase activity as outlined above. For luciferase assay, cells were washed twice with phosphate-buffered saline, dissolved in 250 μl of cell culture lysis reagent (Promega), and harvested by scraping. Luciferase assays were performed immediately using a Promega luciferase assay kit (20). The results are expressed as luciferase activity per μg of protein. All assays were performed in triplicate, and each experiment was repeated a minimum of three times. The triplicates routinely varied by less than 15%. As a control to assure comparable transfection efficiency, we utilized a green fluorescent protein plasmid and determined the percent of cells transfected by visual inspection (21, 32).

**Gel Mobility Shift Assay**—For mobility shift assays, the reaction (20 μl) contained 15% glycerol, 75 mM KCl, 0.375 mM diithiothreitol, 0.375 mM phenylmethylsulfonyl fluoride, 12.5 mM NaCl, 0.1 mg/ml poly(dI-dC), 0.05 mM magnesium acetate, 0.3 μg of nuclear extract, and 0.3 μg of radiolabeled DNA. The mixture was incubated for 5 min at room temperature, and samples were immediately electrophoresed at 250 V for 1.5 h on a 5% nondenaturing polyacrylamide gel using 0.25× TBE running buffer. The gels were then dried for autoradiography. For competition studies, radiolabeled DNA competitor was added as a 20- or 200-fold molar excess. For gel supershift assays, the complete gel mobility shift assay mixture, without the [32P]-labeled oligonucleotide, was incubated at 4 °C for 2 h in the presence of an antibody specific for the C/EBP isoform using 1 μg of rabbit IgG per reaction. Dr. Steven McKnight kindly provided the C/EBPα-specific rabbit polyclonal antibodies generated against amino acids 247–358 of rat C/EBPα (C/EBPα-(247–358)). An additional C/EBPα-specific antibody was obtained from Santa Cruz Biologicals (catalog no. sc-61X). The [32P]-labeled DNA was then added to the mixture and incubated at room temperature for 5 min. The resulting complexes were electrophoresed on a nondenaturing gel for characterization. C/EBPα (sc-150)- and C/EBPβ (sc-636)-specific antibodies were obtained from Santa Cruz Biologicals.

**Immunological Detection of C/EBPα**—Cultured keratinocytes, grown in KSFM, were treated for 24 h with KSFM or with KSFM containing 50 ng/ml TPA before preparation of nuclear extracts as described previously (20, 33). Equal quantities of nuclear protein were electrophoresed on a 10% denaturing polyacrylamide gel and transferred to nitrocellulose. C/EBPα was detected using rabbit anti-human C/EBPα at a dilution of 1:500 (catalog no. sc-61X, Santa Cruz Biologicals) followed by a goat anti-rabbit IgG secondary antibody used at a 1:10,000 dilution. Secondary antibody binding was visualized using chemiluminescent detection reagents.

**RESULTS**

**hINV Promoter C/EBP Site Is Necessary for TPA-dependent Regulation**—A C/EBP transcription factor binding site located in the hINV promoter proximal regulatory region is required for hINV promoter activity (14). Fig. 1 shows that basal promoter activity (open bars) is reduced to 5–10% of control in the absence of a functional C/EBP site. In addition, TPA treatment decreases promoter activity 8-fold; this TPA-dependent activation is absent when the C/EBP site is mutated (solid bars). These results demonstrate a requirement for the C/EBP site for both basal and TPA-activated promoter activity.

**C/EBP Proteins Regulate Promoter Activity**—We next tested the effects of C/EBP transcription factors on promoter activity. Fig. 2A shows that transfection of increasing concentrations of C/EBPα expression plasmid with a constant amount of hINV reporter plasmid increases hINV promoter activity in a concentration-dependent manner in the absence of TPA treatment. Expression is maximally increased by 0.4 μg of C/EBPα plasmid, and the level is not significantly increased at higher concentrations. As different C/EBP heterodimers are known to differentially regulate gene expression, we determined whether C/EBPβ and C/EBPδ can regulate the C/EBPα-dependent activation. As shown in Fig. 2B, C/EBPβ and δ are equally efficient inhibitors of the C/EBPα-dependent activity. In addition, as shown in Fig. 3, the C/EBPα proteins do not regulate promoter activity when the C/EBP-binding site is mutated. These results (i) show that C/EBPα proteins can regulate hINV promoter activity in the absence of TPA treatment, (ii) provide evidence for a dynamic regulatory interaction among C/EBP proteins, and (iii) demonstrate that the proteins act through the C/EBP-binding site.

**GADD153 Inhibits C/EBPα and TPA-dependent Promoter Activity**—To obtain additional evidence of a role for C/EBP factors in TPA-dependent activation, we treated keratinocytes with TPA in the presence of GADD153, a C/EBP family member that inhibits the activity of other C/EBP proteins by inhibiting interaction of the C/EBP-GADD153 complex with DNA (23, 27). This treatment creates an environment in which C/EBP activity is selectively eliminated. As shown in Fig. 4A, transfection of TPA-treated cells with increasing concentrations of GADD153 produces a concentration-dependent inhibition of TPA-dependent promoter activity. As shown in Fig. 2, C/EBPα activates the promoter in the absence of TPA treatment. We would predict that GADD153 would also inhibit the C/EBPα-dependent activation. Fig. 4B shows that GADD153 produces a concentration-dependent inhibition of C/EBPα-dependent activation of promoter activity. In addition, GADD153 produced a concentration-dependent inhibition of basal promoter activity; 0.4 μg of GADD153 expression plasmid/transfection reduced basal activity to 5% of normal (not shown).

**TPA Treatment Increases Binding to the C/EBP Site**—The above described results provide functional evidence suggesting a role for C/EBP proteins as regulators of hINV gene expression. To determine whether TPA treatment alters C/EBP binding, we treated cultures with or without TPA and assayed binding by gel mobility shift assay using the double-stranded hINV C/EBP site oligonucleotide, 5'-GGTTTGCTGCTTTA-GATGCGCTG-3' (C/EBP-binding site in bold). As shown in Fig. 5, gel mobility shift assay indicates that binding to 32P-labeled C/EBP-binding site is increased by 10-fold following treatment with TPA (lanes 1 and 2). The binding is specific, because
addition of a 20- or 200-fold molar excess of radiolabeled C/EBP oligonucleotide inhibits the binding (lanes 3 and 4). No inhibition of binding to 32P-labeled C/EBP oligonucleotide is observed with oligonucleotides encoding consensus AP2 or ets binding sites or a mutant C/EBP-binding site (not shown). In addition, two different C/EBPα-specific antibodies identify the complex prepared from TPA-treated cells as containing C/EBPα (lanes 5 and 6). C/EBPβ- or C/EBPβ-specific antibodies do not detect protein binding (not shown).

**TPA Treatment Does Not Increase C/EBPα Level**—The gel mobility shift experiment indicate that C/EBPα binding to the hINV C/EBP site is increased by TPA treatment. To determine whether this is caused by a change in C/EBPα level, we measured C/EBPα levels in nuclear extracts prepared from untreated and TPA-treated keratinocytes. As shown in Fig. 6, TPA treatment did not increase C/EBPα protein levels.

**The Role of AP1 and C/EBP Sites**—Previous studies indicate that AP1 is an important mediator of TPA-dependent activation (20–22). Because the AP1-binding site is located adjacent to the C/EBP-binding site (34), we wanted to assess the role of C/EBP relative to AP1. We therefore evaluated whether both sites are required for TPA- and C/EBPα-dependent activation of hINV promoter activity. As shown in Fig. 7, mutation of either site results in a loss of TPA- and C/EBPα-dependent activation.
C/EBP complexes differentially regulate gene expression (23–28). This result shows that differential expression of C/EBP factors during keratinocyte differentiation could, in principle, differentially regulate involucrin gene expression.

TPA-dependent Increase in C/EBPα DNA Binding Is Not Associated with an Increase in C/EBPα Concentration—Our studies indicate that the TPA-dependent activation of hINV promoter activity.

FIG. 4. GADD153 inhibits TPA- and C/EBPα-dependent promoter activity. Keratinocytes were cotransfected with pINV-241 (2.5 μg) and increasing concentrations of pGADD153 and then treated with 50 ng/ml TPA (A, B). Cells were cotransfected with 2.5 μg of pINV-241 + 0.5 μg of pC/EBPα and increasing concentrations of pGADD153. The cells were then harvested and assayed for luciferase activity. The values are the average of three separate experiments ± S.E.

FIG. 5. Gel mobility shift analysis of C/EBP-binding site. Nuclear extracts were prepared from near confluence keratinocyte cultures following a 24-h treatment with or without 50 ng/ml TPA. The extracts were then incubated with 32P-labeled double-stranded oligomer, 5′-GGTTTGCTGGTTAAGATGCCTG-3′, containing the hINV C/EBP-binding site. Specificity was demonstrated by including a 20- and 200-fold molar excess of homologous competitor (comp) during the binding reaction. The reactions shown in the two lanes on the right were incubated with two distinct C/EBPα-specific antisera. Complexes were separated by electrophoresis and visualized by autoradiography. FP indicates free probe, and the lower arrowhead indicates C/EBP binding. The arrowhead with the asterisk indicates the supershifted C/EBPα.

FIG. 6. Detection of C/EBPα in keratinocyte nuclear extracts. Nuclear extracts were prepared from keratinocytes after treatment for 24 h in the absence (−) or presence (+) of 50 ng/ml TPA. Equal quantities of protein were electrophoresed, transferred to nitrocellulose, and immunoblotted with a C/EBPα-specific antibody. As a positive control, nuclear extract prepared from C/EBPα expression vector-transfected cells was electrophoresed in a parallel lane (lane C). The arrow indicates the C/EBPα protein, and the numbers to the left of the panel show the molecular mass of marker proteins (kDa). Similar results were observed following an immunoblot of total cell extracts (not shown).

FIG. 7. C/EBPα- and TPA-dependent promoter activation requires the hINV promoter C/EBP and AP1 sites. Keratinocytes were transfected with 2.5 μg of pINV-241, pINV-241(C/EBPm), or pINV-241(AP1-1m) luciferase reporter plasmids in the presence of 50 ng of TPA/ml or 2.5 μg of expression vector encoding C/EBPα. After 24 h, the cells were harvested, and extracts were prepared and assayed for luciferase activity. The results are expressed relative to the activity observed in non-C/EBPα-transfected, non-TPA-treated cultures. The structures of the mutated AP1 and C/EBP sites in pINV(AP1-1m) and pINV(C/EBPm) are described under “Materials and Methods.” The values are the average of four separate determinations, and the bars represent the standard error of the mean.
GADD153 Suppresses C/EBPα and TPA-dependent Transcription—

GADD153 is a unique member of the C/EBP transcription factor family that forms transcriptionally inactive complexes with other C/EBP proteins. GADD153 forms heterodimeric complexes with other C/EBP proteins that are unable to bind to DNA and thus do not regulate transcription (27, 28). Thus, GADD153 can be used selectively to create C/EBP-null conditions (i.e., to selectively knock out C/EBP function). We show that GADD153 inhibits basal transcription. The fact that GADD153 suppresses basal promoter function suggests that endogenous C/EBP proteins are involved in maintaining basal promoter function. When C/EBP levels are artificially elevated by transfection of C/EBPs, GADD153 also inhibits this response, verifying the C/EBP protein requirement for promoter activity. We also show that the TPA-dependent activation of hINV promoter activity is suppressed by GADD153, suggesting that TPA-dependent activation involves signal transduction events that include C/EBP factors. This suggestion is supported by the finding that TPA treatment increases complex formation at the hINV C/EBP-binding site and that C/EBPα is a part of this complex. C/EBPα is the primary C/EBP factor that interacts with the hINV C/EBP site in cells maintained under basal conditions; no binding of C/EBPβ or δ was detected. A possible mechanism to explain the increased promoter activity in response to TPA is that TPA increases C/EBPα binding to the promoter. Consistent with this possibility, gel mobility supershift experiments suggest that C/EBPα is an abundant component of the C/EBP site binding activity present in TPA-treated cells.

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