Expression of CCAAT/Enhancer Binding Proteins (C/EBP) is Associated with Squamous Differentiation in Epidermis and Isolated Primary Keratinocytes and is Altered in Skin Neoplasms

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The epidermis is a stratified squamous epithelium composed primarily of keratinocytes that undergo sequential changes in gene expression during differentiation. CCAAT/enhancer binding proteins (C/EBP) are members of the bZIP family of DNA binding proteins/transcription factors. Northern analysis demonstrated that C/EBPα, C/EBPβ, and C/EBPδ mRNA are expressed in mouse epidermis and their mRNA levels were generally greater than those observed in other tissues known to express high levels of C/EBP. Western analysis of isolated epidermal cell nuclei demonstrated the presence of a 42 and 30 kDa C/EBPα protein and 35 kDa C/EBPβ protein. Immunohistochemical localization of C/EBPα and C/EBPβ in intact interfollicular epidermis revealed that C/EBPβ expression is exclusive to the nuclei of a three-cell cluster of suprabasal keratinocytes that is morphologically consistent with the central column of the epidermal proliferative unit, and that C/EBPα is expressed in the nuclei and cytoplasm of suprabasal keratinocytes and weakly expressed in a perinuclear manner in some basal keratinocytes. In squamous cell carcinomas the expression of C/EBPα and C/EBPβ was greatly diminished as both the intensity of nuclear staining and the number of cells expressing C/EBPα and C/EBPβ were reduced. In isolated primary mouse keratinocytes, calcium-induced differentiation was accompanied by specific temporal changes in the expression of C/EBPα, C/EBPβ, and C/EBPδ mRNA and C/EBPα and C/EBPβ protein. These results implicate a role for the C/EBP family in the regulation of genes involved in or specifically expressed during the process of squamous differentiation in epidermis. ***Key words: cytokines/skin/transcription factors. J Invest Dermatol 110:939–945, 1998***

The epidermis is a stratified squamous epithelium composed primarily of keratinocytes that undergo a highly coordinated program of sequential changes in gene expression during differentiation from proliferating basal cells through morphologically distinct suprabasal cells, ending in the nonviable cornified stratum corneum (Watt, 1989; Fuchs, 1990). The expression of catalytic proteins such as transglutaminase (Thacher and Rice, 1985), assembly proteins such as filaggrin (Dale *et al*, 1985), and structural proteins such as loricrin (Mehrel *et al*, 1990), keratins (Fuchs and Green, 1980), involucrin (Simon and Green, 1984), and cornifin-α/SPRR1 (Gibbs *et al*, 1993; Owens *et al*, 1996), is strictly regulated as a function of the stage of keratinocyte differentiation. The induction of certain differentiation responsive genes is tightly coupled with the repression of others during differentiation. In addition to undergoing a precisely defined program of differentiation, keratinocytes are a rich source of numerous cytokines such as IL-1α, IL-6, IL-7, IL-8, granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor-α that can regulate the function of cells in skin (for review, see Matsue *et al*, 1992). These epidermal cytokines can act as autocrine and paracrine factors that may play a role in amplifying responses of the skin to various stimuli such as chemicals and UV light. Although cytokine expression in keratinocytes and the coordinate and sequential alterations in specific genes involved in keratinocyte differentiation have been well characterized, the transcription factors that regulate these processes in keratinocytes are largely uncharacterized.

CCAAT/enhancer binding protein α (C/EBPα) is a heat-stable transcription factor that recognizes consensus sequence (5′-ATTGCGCAAT-3′) within many promoters (Vinson *et al*, 1989; Johnson, 1993). The C/EBP family contains at least seven members that include C/EBPα, C/EBPβ (also termed NF-IL6, NF-M, IL-6DBP, CRP2, or LAP), C/EBPδ (NF-IL6β or CRP3), C/EBPγ (Ig/EBP-1), CRP1, d/CEBP, and CHOP10 (GADD153) (for review, see Wedel and Loms Ziegler-Heitbrock, 1995). C/EBP proteins are members of the bZIP family of DNA-binding proteins and consist of three structural components: a *C*-terminal leucine zipper, a basic DNA-binding region (Landschulz *et al*, 1988; Vinson *et al*, 1989), and an *N*-terminal transactivating region (Friedman and McKnight, 1990). C/EBP proteins share amino acid sequence similarities within their *C*-terminal basic region/leucine zipper domain. The leucine zipper domain is responsible for dimerization, whereas the basic region is responsible for binding to specific DNA sequences (Vinson *et al*, 1989). Both homo- and heterodimers of C/EBP isoforms can form and bind to C/EBP sites within the promoters/enhancers of certain genes (Friedman *et al*, 1989; Descombes *et al*, 1990; Cao *et al*, 1991; Williams *et al*, 1991).

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Abbreviations: C/EBP, CCAAT/enhancer binding protein; EPU, epidermal proliferative unit.

The expression of C/EBP isoforms is most prominent in adipocytes, hepatocytes, intestinal tissues, lung (Birkenmeier *et al*, 1989; Cao *et al*, 1991), monocytes/macrophage (Natsuka *et al*, 1992), and ovarian follicles (Piontkewitz *et al*, 1993). C/EBPα is produced in tissues capable of gluconeogenesis and lipogenesis, especially liver and fat (Birkenmeier *et al*, 1989), and it is considered to play a direct role in regulating transcription of some of the enzymes involved in controlling these metabolic processes (McKnight *et al*, 1989). Many lines of evidence indicate that C/EBPα plays a key role in the differentiation of preadipocytes into adipocytes as it appears to function both by inhibiting the clonal expansion that precedes adipocyte terminal differentiation and by activating the coordinate expression of a group of adipocyte genes whose promoters possess C/EBP-binding sites (Christy *et al*, 1989; Cao *et al*, 1991; Umek *et al*, 1991; Freytag *et al*, 1994). Recent studies indicate that C/EBPα inhibits cell proliferation of fibrosarcoma cells through the upregulation of the cyclin-dependent kinase inhibitor, p21 (Timchenko *et al*, 1996).

C/EBPβ plays a role at the early stages of preadipocyte differentiation and leads to the expression of C/EBPα, after which C/EBPβ is downregulated as the adipocytes acquire the complete fat-specific phenotype (Cao *et al*, 1991; Yeh *et al*, 1995). C/EBPβ is more widely expressed than C/EBPα, e.g., C/EBPβ expression increases during differentiation of macrophage, myeloid, and plasma cells (Cooper *et al*, 1992; Natsuka *et al*, 1992; Scott *et al*, 1992), suggesting its involvement in the differentiation of these lineages. Moreover, there is evidence that C/EBPβ is involved in the regulation of the expression of several cytokines and C/EBPβ binding motifs are found in the regulatory regions of IL-1β, IL-6, IL-8, tumor necrosis factor-α, and granulocyte-colony stimulating factor (G-CSF) (Akira *et al*, 1990; Mukaida *et al*, 1990; Drouet *et al*, 1991; Natsuka *et al*, 1992; Zhang and Rom, 1993). Like C/EBPβ, C/EBPδ also appears to play a role at the early stages of preadipocyte differentiation (Cao *et al*, 1991; Yeh *et al*, 1995). Recent studies have demonstrated that C/EBPδ is important for regulating the acute phase expression of the human C3 (the third component of complement) gene in the presence of IL-1 (Juan *et al*, 1993), suggesting a role of C/EBPδ in the regulation of several IL-1 target genes.

As C/EBP proteins play a fundamental role in the differentiation of preadipocytes to adipocytes and in the regulation of the expression of many different genes encoding cytokines in several cell types, we reasoned that C/EBP may be expressed in epidermis and they may play an important role in differentiation and/or cytokine expression. Our results demonstrate that C/EBPα, C/EBPβ, and C/EBPδ mRNA are highly expressed in mouse epidermis and in primary keratinocytes. Furthermore, C/EBPα and C/EBPβ proteins are preferentially expressed in specific suprabasal keratinocytes in epidermis and their expression is upregulated during calcium-induced differentiation of primary keratinocytes.

MATERIALS AND METHODS

Materials Antibodies for C/EBP isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Keratin 1 polyclonal antibody was purchased from Berkeley Antibody Company (Richmond, CA). Biotinylated secondary goat anti-rabbit IgG was purchased from Boehringer Mannheim (Indianapolis, IN). Peroxidase-conjugated streptavidin and 5,5′-diaminobenzidine were purchased from BioGenex (San Ramon, CA). Protease inhibitors, sodium orthovanadate, and DNAse were purchased from Sigma (St. Louis, MO). [α-^{32}P]dCTP was purchased from DuPont-New England Nuclear Research Products (Boston, MA). Fetal bovine serum and trypsin were purchased from GIBCO BRL (Gaithersburg, MD). EMEM (Ca^{++} free) was purchased from BioWhittaker (Walkersville, MD). Epidermal growth factor was purchased from United States Biochemical (Cleveland, OH).

Animals Female CD-1 mice, 6–7 wk old, or pregnant CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC). The mice were kept in our facility for 1 wk prior to use and were fed rodent chow (Agway Food, Granville Milling, Creedmore, NC) and water *ad libitum*. The mice were kept on corn cob bedding and placed on a 12 h light/dark cycle until they were used. The hair of the dorsal skin of 6–7 wk old mice was clipped with electric clippers.

RNA isolation and northern blot analysis Epidermis was removed from the dermis (Goodell *et al*, 1996) and total RNA was isolated from the epidermis/other tissues or from primary mouse keratinocytes using the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction as described previously (Chomczynski and Sacchi, 1987). Thirty micrograms of each RNA sample was subjected to electrophoresis on a 1% agarose-formaldehyde gel, transferred to a Zeta-probe^{GT} nylon membrane (Bio-Rad, Hercules, CA), and prehybridized at 65°C for 10 min in a solution containing 0.25 M sodium phosphate buffer (pH 7.2) and 7% sodium dodesyl sulfate. Hybridization was conducted overnight at 65°C with 25 ng of ^{32}P-labeled C/EBPα rat cDNA isolated from the pMSV-C/EBPα plasmid, a gift from Dr. S. L. McKnight (University of Texas Southwestern Medical Center, Dallas, TX). After hybridization, the membrane was washed and exposed to Kodak X-OMAT AR film in a cassette with intensifying screens at –80°C. The same membrane was stripped and reprobed with radiolabeled cornifin-α/SPRR1 cDNA, a gift from Dr. A.M. Jetten (National Institute of Environmental Health Sciences, Research Triangle Park, NC), 7S RNA cDNA, a gift from Dr. A. Balmain (The Beatson Institute for Cancer Research, Glasgow, U.K.), or polymerase chain reaction cloned mouse genes corresponding to C/EBPβ and C/EBPδ. Mouse genomic DNA was utilized to polymerase chain reaction clone the intronless C/EBPβ and C/EBPδ genes using the following forward and reverse primer for C/EBPβ, 5′-TTCTACTACGAGCCCGACTGCC-3′ and 5′-CAGCTTGTCCACCGTCTTCTTG-3′, respectively, and the following forward and reverse primers for C/EBPδ, 5′-CCAGATTTTCATTTCGCTCCAG-3′ and 5′-TCGCAGGTCCCAAAGAAACTAG-3′, respectively. The identity of C/EBPβ and C/EBPδ polymerase chain reaction products was confirmed by the size of the amplified product and restriction enzyme analysis. 7S RNA cDNA was used to confirm that equal amounts of RNA were loaded (Balmain *et al*, 1982). All cDNA were radiolabeled by random priming using a random priming kit (GIBCO BRL) and [α-^{32}P]dCTP (3000 Ci per mmol, 10 μCi per μl) and purified from unincorporated [α-^{32}P]dCTP using Push Columns (Stratagene, La Jolla, CA).

Nuclei isolation from epidermal cells and western blot analysis The preparation of epidermal cells from mouse skin was performed as described by Goodell *et al* (1996). The epidermal cell nuclei were isolated as described by Chapin *et al* (1994). The nuclei were resuspended in RIPA buffer [phosphate buffered saline (PBS) containing 1% Nonidet P-40, 0.5% sodium deoxycholic acid, and 0.1% sodium dodecyl sulfate] with protease inhibitors (100 μg aprotinin per ml and 0.1 mM phenylmethylsulfonyl fluoride) and tyrosine phosphatase inhibitor (1 mM sodium orthovanadate). The resuspension was sonicated, incubated on ice for 1 h, and subjected to centrifugation for 20 min at 4°C, 11,000 × *g*. In some experiments, epidermal cells or primary keratinocytes were placed in the above buffer, sonicated, incubated on ice for 1 h, and subjected to centrifugation for 20 min at 4°C, 11,000 × *g*. The supernatant was stored at –80°C prior to analysis and the protein concentration was determined using the Lowry method (Lowry *et al*, 1951). Epidermal homogenate, C/EBPα and C/EBPβ standard proteins, and molecular weight markers (GIBCO BRL) were boiled in sodium dodecyl sulfate sample loading buffer for 4 min and subjected to reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis through Tris-Glycine gels. The C/EBPα and C/EBPβ standard proteins were gifts from Drs. Peter Johnson and Esta Sterneck (National Cancer Institute, Bethesda, MD). The separated proteins were electrophorectically transferred to an Immobilon P membrane (Millipore, Bedford, MA). Following incubation in blocking buffer (PBS with 1% bovine serum albumin, 5% milk, and 0.1% Tween) for 1 h at room temperature, the membranes were probed overnight at 4°C with rabbit polyclonal IgG raised against C/EBPα (1:5000), C/EBPβ (1:2500), keratin 1 AF109 (1:500), or cornifin-α/SPRR1 (1:5000), a gift from Dr. A.M. Jetten (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The membranes were then probed with a horseradish peroxidase-linked donkey anti-rabbit Ig (Amersham, Arlington Heights, IL) for 1 h at room temperature. Detection was made with a luminol system (ECL system, Amersham), and the resulting light was then recorded on to Kodak BioMax MR film. Upon completion of autoradiography, membranes were stained with amido black to confirm equal protein content in all the lanes.

Immunohistochemical localization Skin samples (n = 5), 7,12-dimethylbenz[a]anthracene-initiated/12-*O*-tetradecanoylphorbol-13-acetate-promoted squamous papillomas (n = 8), and squamous cell carcinomas (n = 4) were frozen in OCT compound (Miles, Elkhart, IN). Frozen sections (5 μm) on Super Frost Plus slides (Fisher, Pittsburgh, PA) were air dried for 30 min at room temperature and fixed in ice-cold acetone for 10 min on ice followed by rinsing in three changes of PBS. The endogenous peroxidase activity was quenched by incubation in 0.1% H_2O_2 in PBS for 10 min at room temperature. After rinsing in three changes of PBS, the nonspecific binding of antibodies was blocked by incubating the sections in 1% normal goat serum in PBS for

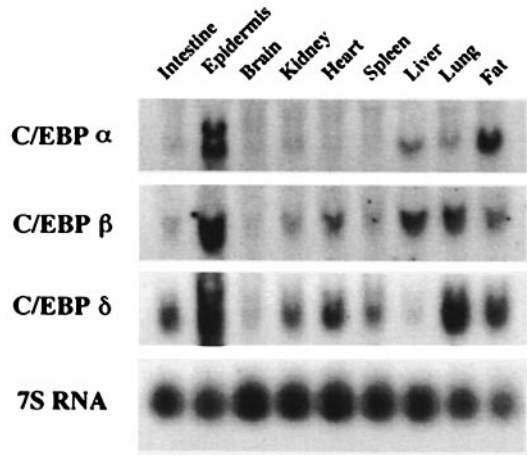

Figure 1. C/EBPα, C/EBPβ, and C/EBPδ mRNA are abundantly expressed in mouse epidermis. Total cellular RNA was isolated from the epidermis and several other tissues and northern blot analysis was conducted. C/EBPα, C/EBPβ, and C/EBPδ mRNA sizes were 2.7, 2.0, and 1.8 kb, respectively. The membrane was reprobed with 7S RNA cDNA to verify the equal loading of RNA.

30 min at room temperature. The excessive blocking solution was drained and the sections were then incubated with the primary polyclonal antibodies against C/EBPα (1:25,000) and C/EBPβ (1:10,000) in 1% bovine serum albumin in PBS at 4°C overnight. After washing with PBS, the samples were incubated with a biotinylated goat-anti-rabbit IgG for 30 min at room temperature followed by a 30 min incubation with peroxidase conjugated streptavidin. The avidin/biotin-peroxidase complexes were visualized by incubation with 5,5′-diaminobenzidine (following the manufacturer's protocol). The sections were counterstained lightly with Harris-modified hematoxylin, dehydrated, and permounted with cover glasses. No C/EBP isoform staining was observed when the primary antibody was omitted and the control rabbit serum was applied.

Isolation and culture of primary keratinocytes Primary keratinocytes were isolated from newborn CD-1 mice (1–2 d old) by trypsin floatation (Hennings *et al*, 1980). Isolated epidermal cells were plated at 6×10^6 cells per 60 mm plate in Ca^{++} free EMEM supplemented with 10% fetal bovine serum and 4 ng epidermal growth factor per ml for 4 h to enhance keratinocyte attachment. Cells were then gently washed with Mg^{++} and Ca^{++} free PBS twice to remove any remaining calcium and then cultured in Ca^{++} free EMEM supplemented with 8% chelex-treated fetal bovine serum and 4 ng epidermal growth factor per ml. Calcium chloride was then added to produce a final concentration of 0.05 mM Ca^{++}. After 3 d in culture, calcium chloride was added to produce a final concentration 0.12 mM Ca^{++} or 1.5 mM Ca^{++} (Yuspa *et al*, 1989). At various times, keratinocytes were harvested for the isolation of protein and RNA for western and northern analysis, respectively.

RESULTS

C/EBPα, C/EBPβ, and C/EBPδ are abundantly expressed in mouse epidermis In order to determine whether C/EBPα, C/EBPβ, and C/EBPδ mRNA are expressed in mouse epidermis, total RNA was isolated from the epidermis and northern blot analysis was conducted. For comparison purposes, northern analysis was also conducted on total RNA isolated from several other organs or tissues known to express specific isoforms of C/EBP (Birkenmeier *et al*, 1989; Cao *et al*, 1991; Williams *et al*, 1991). As shown in **Fig 1**, C/EBPα, C/EBPβ, and C/EBPδ mRNA are expressed in the epidermis with an approximate size of 2.7, 2.0, and 1.8 kb, respectively. Close inspection revealed two bands (≈2.7–3.0 kb) that hybridized with the C/EBPα cDNA, as the lower band more closely approximates the size of C/EBPα transcripts in other tissues we only made comparisons to this band. When C/EBPα mRNA levels in epidermis were compared with mRNA levels in liver, lung, and fat, three tissues known to express high levels of C/EBPα, epidermal C/EBPα mRNA levels were higher than liver and lung and similar to that observed in fat. When the levels of C/EBPβ and C/EBPδ mRNA were compared with mRNA levels of C/EBPβ and C/EBPδ in other tissues known to express high levels of these isoforms, the mRNA levels of C/EBPβ and C/EBPδ were expressed at their highest level in the epidermis.

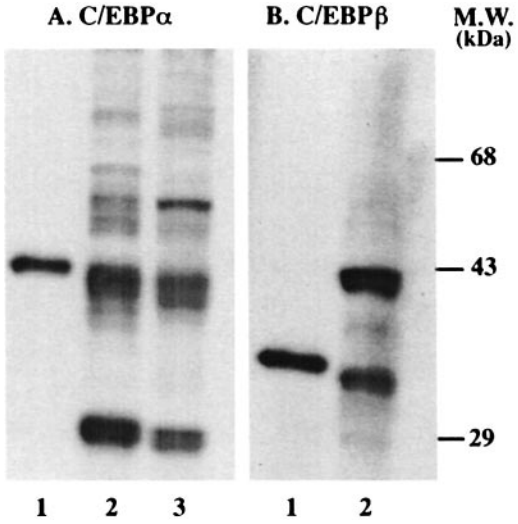

Figure 2. Expression of C/EBPα and C/EBPβ proteins in the mouse epidermis. Epidermal cells were isolated from mouse skin by trypsinization. Whole homogenate or nuclei extract was prepared and western blot analysis was conducted using isoform specific rabbit polyclonal antibodies made to rat C/EBPα amino acids 253–265 and rat C/EBPβ amino acids 258–276. (*A*) *Lane 1*, C/EBPα standard protein; *lane 2*, epidermal nuclei extract, 20 μg; *lane 3*, epidermal homogenate, 40 μg. (*B*) *Lane 1*, C/EBPβ standard protein; *lane 2*, epidermal nuclei extract, 20 μg.

To assure that C/EBPα, C/EBPβ, and C/EBPδ are expressed at the protein level, epidermal cells were isolated from mouse skin by trypsinization, nuclei were isolated, and western blot analysis was conducted using isoform specific antibodies. As shown in **Fig 2**(***A***), the C/EBPα specific antibody recognized a 42 and 30 kDa protein in the epidermal nuclei preparation (*lane 2*) as well as in the epidermal homogenate (*lane 3*). Based on the molecular weight of these proteins and the known molecular weights of C/EBPα (Wedel and Loms Ziegler-Heitbrock, 1995), as well as the mobility of the C/EBPα standard (histidine-tagged bacterially expressed protein runs slightly slower), we have classified these proteins as C/EBPα p42 and C/EBPα p30. In addition, C/EBPα p42 was detected as a doublet, suggesting the possibility of post-translational modification of C/EBPα protein by phosphorylation. Minor bands are not visible when exposure times are decreased. As shown in **Fig 2**(***B***), a 35 kDa protein was detected in the epidermal nuclei preparation by the C/EBPβ specific antibody. This protein comigrated with the C/EBPβ standard (histidine-tagged bacterially expressed protein runs slightly slower), and we have classified this 35 kDa protein as C/EBPβ. The identity of the higher molecular weight band (42 kDa) is unknown; however, it likely represents binding of the primary antibody as incubation with the secondary antibody alone did not produce this band. In addition, this 42 kDa protein is present in C/EBPβ knockout mouse epidermis (Sterneck *et al*, 1997), although the 35 kDa C/EBPβ protein is absent (data not shown). These results indicate that the 42 kDa protein is not related to the expression of C/EBPβ. Finally, we were not able to detect C/EBPδ protein with a commercial C/EBPδ specific antibody. As other antibodies to C/EBPδ are not available, we do not know if the C/EBPδ protein is not expressed or if the antibody we utilized failed to recognize C/EBPδ protein.

C/EBPα and C/EBPβ each display a unique cellular and subcellular distribution within specific keratinocytes in intact epidermis, and their expression is altered in skin neoplasms In order to determine which cells of the epidermis express C/EBPα and C/EBPβ, immunohistochemical localization was conducted on intact frozen skin sections. As shown in **Fig 3**(***A***), C/EBPα was expressed in the nuclei and cytoplasm of suprabasal keratinocytes and was weakly expressed in a perinuclear manner in some basal keratinocytes. No detectable C/EBPα staining was observed in the stratum corneum. Also, we have conducted competition experiments in which we preincubated the C/EBPα antibody with bacterially expressed

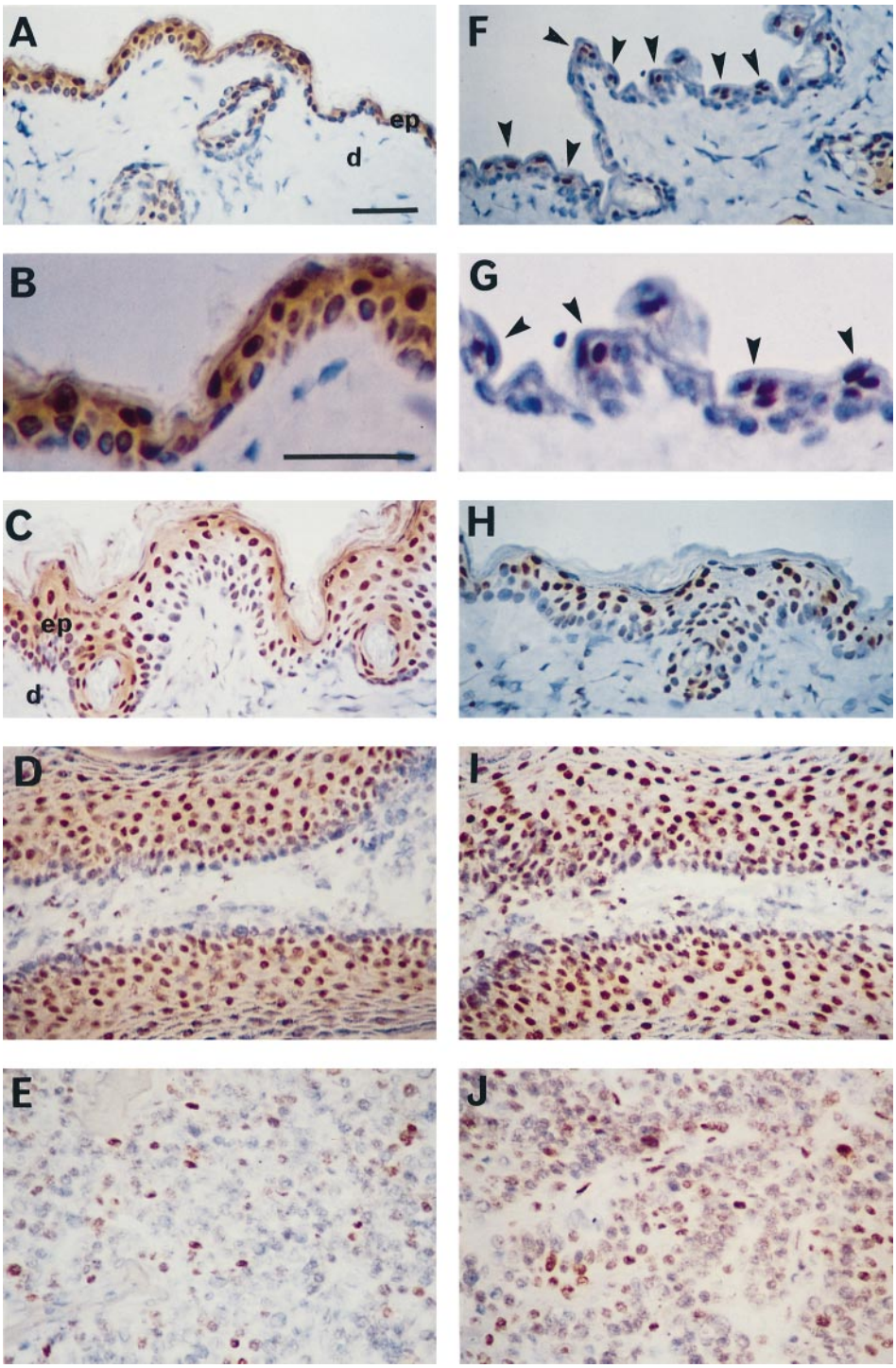

Figure 3. C/EBPα and C/EBPβ display a unique cellular and subcellular distribution within specific keratinocytes in intact epidermis and are altered in skin neoplasms. The intact sections of control and 12-O-tetradecanoylphorbol-13-acetate treated (5 nmol, 2X) skins and neoplasms were stained for C/EBPα and C/EBPβ as described in *Materials and Methods*. (*A*) C/EBPα in normal epidermis; (*B*) C/EBPα in normal epidermis (higher magnification); (*C*) C/EBPα in TPA treated epidermis; (*D*) C/EBPα in squamous papilloma; (*E*) C/EBPα in squamous cell carcinoma; (*F*) C/EBPβ in normal epidermis; (*G*) C/EBPβ in normal epidermis (higher magnification); (*H*) C/EBPβ in TPA treated epidermis; (*I*) C/EBPβ in squamous papilloma; (*J*) C/EBPβ in squamous cell carcinoma. No C/EBP isoform staining was observed when the primary antibody was omitted or when the control rabbit serum was applied. The *arrowheads* indicate the nuclei expression of C/EBPβ in three-cell clusters at each EPU in normal epidermis. ep, epidermis; d, dermis. *Scale bars*, 10 μm.

C/EBPα protein or the C/EBPα peptide used to make the antibody, and then carried out immunohistochemical staining of the epidermis. When the C/EBPα antibody was preincubated with the bacterially expressed C/EBPα protein or the C/EBPα peptide, it did not produce any staining in the epidermis, whereas the control antibody not preincubated with the bacterially expressed C/EBPα protein or the C/EBPα peptide produced the characteristic C/EBPα staining pattern (data not shown). **Figure 3**(***B***) shows higher magnification of C/EBPα staining in the epidermis. Next, we examined C/EBPα expression in hyperplastic epidermis in an attempt to better localize its expression as well as to examine its expression under conditions of non-neoplastic cell growth. As shown in **Fig 3**(***C***), in hyperplastic epidermis C/EBPα was localized to the nuclei of spinous and granular keratinocytes with no detectable staining in the stratum corneum and little to no staining in basal keratinocytes. In squamous papillomas the expression of C/EBPα was similar to that observed in hyperplastic epidermis (**Fig 3*D***); however, in squamous cell carcinomas C/EBPα expression was greatly diminished as both the intensity of nuclear staining and the number of cells expressing C/EBPα were reduced (**Fig 3*E***). In addition, the specific pattern of expression observed in the epidermis and squamous papillomas was absent and the cells expressing C/EBPα appeared randomly distributed.

C/EBPβ expression demonstrated a highly ordered and specific pattern in the epidermis. C/EBPβ expression was exclusive to the nuclei of a three-cell cluster of suprabasal keratinocytes and these intensely stained clusters were repeated at regular intervals throughout

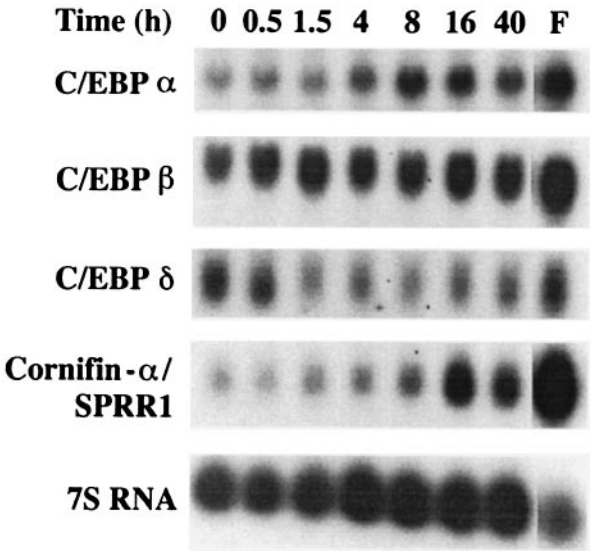

Figure 4. C/EBPα, C/EBPβ, and C/EBPδ mRNA are altered in primary keratinocytes induced to differentiate by increasing extracellular calcium concentration. Mouse keratinocytes were isolated as described in the *Materials and Methods*. Keratinocytes were plated in media containing 0.05 mM Ca^{++} cultured for 3 d and switched to media containing 0.12 mM Ca^{++}. Keratinocytes were harvested at the indicated times (in hours) after the calcium switch and RNA was extracted and northern analysis conducted. F, floating cells that were collected on day 2 after the initial plating. Although 7S RNA is increasing with time, densitometric scanning of the photographic negative of the ethidium bromide stained 18S or 28S RNA before RNA was transferred to membrane did demonstrate equal loading.

the epidermis (**Fig 3*F***). This characteristic pattern is morphologically consistent with the central suprabasal column of the epidermal proliferative unit (EPU) (Potten, 1974; Morris *et al*, 1985). No detectable C/EBPβ staining was observed in the stratum corneum. When immunohistochemical staining experiments were conducted on the C/EBPβ knockout mouse skin using the above C/EBPβ antibody, we found no immunohistochemical staining in the skin (data not shown). **Figure 3(*G*)** shows higher magnification of C/EBPβ staining of the three-cell cluster of suprabasal keratinocytes. In hyperplastic epidermis the highly ordered expression pattern of C/EBPβ observed in normal epidermis was absent; however, C/EBPβ was expressed in the nuclei of the suprabasal keratinocytes with no detectable expression in basal keratinocytes (**Fig 3*H***). In squamous papillomas the expression of C/EBPβ was similar to that observed in hyperplastic epidermis (**Fig 3*I***); however, in squamous cell carcinomas C/EBPβ expression was greatly diminished (both staining intensity and number of cells) and there was a loss of the expression patterns observed in squamous papillomas or epidermis (**Fig 3*J***).

C/EBPα and C/EBPβ expression is increased in primary mouse keratinocytes induced to differentiate by high extracellular calcium concentrations Basal mouse primary keratinocytes can be cultured in medium containing 0.05 mM Ca^{++}; however, the terminal differentiation can be induced by increasing the level of extracellular calcium in the media (Hennings *et al*, 1980). We wanted to determine whether calcium-induced differentiation of primary mouse keratinocytes is associated with alterations in the temporal expression of C/EBP isoforms. We chose to examine the effect of two extracellular calcium concentrations (0.12 mM and 1.5 mM Ca^{++}) on the expression of C/EBP isoforms as the expression of certain differentiation markers in mouse keratinocytes is coupled to the level of extracellular calcium concentrations (Yuspa *et al*, 1989). Primary mouse keratinocytes were cultured in medium containing 0.05 mM Ca^{++} for 3 d and then switched to either 0.12 mM or 1.5 mM Ca^{++}. The switch to 0.12 and 1.5 mM Ca^{++} was associated with changes in keratinocyte morphology, characteristic of a more differentiated phenotype (Hennings *et al*, 1980), that were apparent at 8 and 1.5 h, respectively. As shown in **Fig 4**, the switch to 0.12 mM Ca^{++} was accompanied by

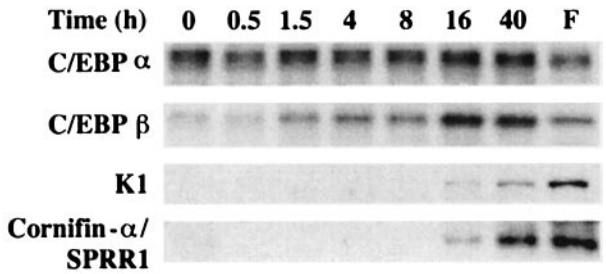

Figure 5. C/EBPα and C/EBPβ proteins are altered in primary keratinocytes induced to differentiate by increasing extracellular calcium concentration. Mouse keratinocytes were isolated as described in the *Materials and Methods*. Keratinocytes were plated in media containing 0.05 mM Ca^{++} cultured for 3 d and switched to media containing 0.12 mM Ca^{++}. Keratinocytes were harvested at the indicated times (in hours) after the calcium switch and proteins were analyzed by western analysis. F, floating cells that were collected on day 2 after the initial plating.

changes in the expression of C/EBPα, C/EBPβ, C/EBPδ, and cornifin-α/SPRR1 mRNA. C/EBPα mRNA was maximally increased at 8 and 16 h, followed by a decrease at 40 h. C/EBPβ mRNA levels were only slightly increased at all time points after the switch to 0.12 mM Ca^{++}, whereas C/EBPδ levels were decreased at all time points after the switch. Cornifin-α/SPRR1 mRNA was maximally increased at 16 and 40 h after the switch to 0.12 mM Ca^{++}. Floating cells, presumably differentiated keratinocytes, were collected on days 1 and 2 after the initial plating, as shown in **Fig 4**, northern analysis of floating cells collected on day two demonstrated high levels of C/EBPα, C/EBPβ, and cornifin-α/SPRR1 mRNA expression. Western analysis demonstrated modest increases in C/EBPα protein at 16 and 40 h of 20% as determined by lasar densitometric scanning of exposed film, whereas C/EBPβ protein was increased 2-fold by 1.5 h and 7–8-fold at 16 and 40 h (**Fig 5**). Both keratin 1 and cornifin-α/SPRR1 protein could be detected at 16 and 40 h and both were expressed at high levels in the floating cells. Keratinocytes that were not switched to high calcium containing medium, but that continued to be cultured in 0.05 mM Ca^{++}, did not demonstrate changes in C/EBPα, C/EBPβ, cornifin-α/SPRR1, and K1 from the levels observed at time zero (data not shown).

Western analysis of proteins isolated from keratinocytes switched to 1.5 mM Ca^{++} medium were similar to the results obtained with 0.12 mM Ca^{++}, with the exception that C/EBPα protein remained at control levels until 4 h and then decreased at 8 and 16 h and was barely detectable at 40 h, and that C/EBPβ and cornifin-α/SPRR1 levels were increased at one time point earlier than that observed in 0.12 mM Ca^{++} medium and keratin 1 protein could not be detected (data not shown).

DISCUSSION

The results presented in this paper demonstrate that the bZIP transcription factors C/EBPα, C/EBPβ, and C/EBPδ are abundantly expressed at the mRNA level in mouse epidermis and C/EBPα and C/EBPβ are expressed in specific subpopulations of suprabasal keratinocytes in intact epidermis. It has been proposed that the epidermis is organized into units, referred to as the EPU (Potten, 1974). Our finding that C/EBPβ expression is exclusive to the nuclei of a three-cell cluster of suprabasal keratinocytes, and that these intensely stained clusters are repeated at regular intervals throughout the epidermis, suggests that these cells are morphologically consistent with the differentiative column of the EPU (Potten, 1974; Morris *et al*, 1991). The EPU is composed of a suprabasal column or a stack of three nucleated cells that exhibit a flat hexagonal morphology that covers an area of ≈10 basal cells below it. Within this hexagonal area of 10 basal keratinocytes, a centrally located keratinocyte has been identified that is a slow cycling cell and is considered to have an unlimited capacity for self-renewal, whereas the remaining basal cells of the EPU are either transient amplifying cells with a limited capacity for self-renewal or post-mitotic daughter cells that have no proliferative capacity (Potten, 1974). Within

the EPU, differentiation is strictly coordinated with proliferation and involves the movement of a post-mitotic basal keratinocyte to the suprabasal layer, this process eventually results in a column or stack of three post-mitotic suprabasal keratinocytes in various stages of terminal differentiation. The highly specific and ordered expression of C/EBPβ suggests that C/EBPβ plays an integral role within the cells of the EPU. Another bZIP protein, c-Fos, which has been proposed to function in the terminal stages of epidermal differentiation, also exhibits exclusive expression in the three cells of the EPU (Fisher *et al*, 1991). Fos and C/EBP can form heterodimers *in vitro* (Hsu *et al*, 1994) that could impart another level of specificity to the regulation of differentiation responsive genes.

It is intriguing that the highly ordered specific expression of C/EBPβ is absent in phorbol ester-induced hyperplastic epidermis, papillomas, and squamous cell carcinomas, indicating that the deregulated keratinocyte growth is accompanied by loss of the ordered C/EBPβ-EPU structure. This concept is supported by Morris *et al* (1985), who provided evidence that phorbol ester causes the premature vertical migration of basal keratinocytes located at the periphery of the EPU and the proliferation of the centrally located slow cycling cell of EPU. The consequence of the loss of C/EBPα and C/EBPβ expression in the squamous cell carcinomas is not understood; however, it is known that keratin 1, keratin 10, loricrin, and filaggrin are absent or greatly reduced in squamous cell carcinomas (Yuspa *et al*, 1991). C/EBP binding sites are found in the promoter regions of keratin 1, keratin 10, and involucrin. Further study will be required to determine if there is any relationship between the alteration in the expression of these genes in squamous cell carcinomas and loss of C/EBPα and C/EBPβ expression.

The vast majority of C/EBPα present in the epidermis is in the nuclei and cytoplasm of suprabasal cells, and we conclude that these cells represent suprabasal keratinocytes based on the fact that the keratinocyte is the predominate cell type in the epidermis (≈95%) and C/EBPα is expressed in primary mouse keratinocytes. In addition, recent reports have demonstrated that C/EBPα and C/EBPβ are expressed in cultured human foreskin (Wang *et al*, 1996) and that C/EBPα is expressed in adult keratinocytes and in suprabasal cells of human epidermis (Swart *et al*, 1997). At this time we do not know the identity of the few basal cells that express C/EBPα; however, these cells may represent post-mitotic keratinocytes committed to differentiation and to the eventual upward movement to the suprabasal layers, or a minor epidermal cell type such as Langerhans cells or melanocytes. The fact that C/EBPα is expressed in isolated keratinocytes cultured in 0.05 mM Ca^{++} medium suggests that it has a function in basal keratinocytes.

Our finding that calcium-induced differentiation of primary keratinocytes is associated with increases in C/EBPα and C/EBPβ protein, provides further evidence for a role for C/EBP isoforms in the regulation of genes that are specifically expressed during squamous differentiation or genes involved in the process of differentiation. It is clear that C/EBPβ is highly induced in medium containing 0.12 mM Ca^{++}, whereas C/EBPα is modestly induced. As C/EBP isoforms can form homodimers as well as heterodimers it is possible that the large increase in C/EBPβ allows for heterodimerization with C/EBPα, which in itself has a different regulatory function than either C/EBPα or C/EBPβ homodimers. The heterodimerization between different C/EBP isoforms, as well as interactions between C/EBP isoforms and other transcription factors such as AP-1 (Jun/Fos) (Hsu *et al*, 1994) and NF-κB (Stein *et al*, 1993), could provide an additional level of complexity in determining the functional properties of this group of transcription factors. Our observations that different levels of high calcium (0.12 *vs* 1.5 mM Ca^{++}) have different effects on the temporal expression and level of expression of C/EBPα, C/EBPβ, keratin 1, and cornifin-α/SPRR1, is consistent with the results of Yuspa *et al* (1989) who demonstrated that the expression of certain differentiation markers in mouse keratinocytes is coupled to the level of extracellular calcium concentrations. For example, Yuspa *et al* (1989) demonstrated that spinous and granular markers are expressed in mouse keratinocytes in media containing 0.12 mM Ca^{++}, but higher levels of Ca^{++} such as those ⩾ 1 mM cause a rapid differentiation in which granular markers are rapidly expressed but spinous markers such as keratin 1 are not expressed. Differences in C/EBP isoform expression in media containing 0.05, 0.12, and 1.5 mM Ca^{++} indicate that the expression of these proteins is altered by different levels of calcium, and as such their expression may be related to the calcium gradient found in intact epidermis.

It is apparent that the levels of C/EBPα and C/EBPβ mRNA do not always coincide with the levels of their encoded proteins, which suggests that C/EBP protein expression is also controlled at post-transcriptional and/or translational levels. This is most evident in the floating cells where high levels of C/EBPα and C/EBPβ mRNA are present; however, protein levels are not correspondingly elevated. The discrepancy between mRNA levels and protein levels of C/EBP isoforms has been observed in other cell types and tissues (Birkenmeier *et al*, 1989; Descombes *et al*, 1990; Williams *et al*, 1991) and further supports the hypothesis that the expression of C/EBP proteins are regulated at post-transcriptional and/or translational levels. Finally, in primary keratinocytes we found only one C/EBPα message and only the C/EBPα p42 kDa protein, whereas in epidermal preparations from intact epidermis we found two C/EBPα messages as well as C/EBPα p42 and C/EBPα p30 proteins. Whether the second mRNA species and the 30 kDa protein are related is not known.

Keratinocytes express numerous cytokines such as IL-1α, IL-6, IL-7, IL-8, GM-CSF, and tumor necrosis factor-α (for review, see Matsue *et al*, 1992). Epidermal keratinocytes are a vast reservoir of IL-1 (Schmitt *et al*, 1986) and higher levels of IL-1α protein are associated with suprabasal keratinocytes rather than with the basal keratinocytes in mouse epidermis (Lee *et al*, 1994). In addition to IL-1α, it has been shown that GM-CSF protein is localized to suprabasal keratinocytes within the epidermis (Robertson *et al*, 1994). C/EBPβ is involved in the regulation of the expression of several cytokines (Akira *et al*, 1990; Mukaida *et al*, 1990; Drouet *et al*, 1991; Natsuka *et al*, 1992; Zhang and Rom, 1993). Therefore, it is possible that C/EBP proteins may be involved in the regulation of the expression of cytokines within suprabasal keratinocytes, because C/EBPα and C/EBPβ and certain cytokines are predominately expressed in these cells.

Although further studies are required to elucidate the genes that C/EBP isoforms regulate in keratinocytes, our study provides new insights and novel information regarding C/EBP isoform expression in specific suprabasal keratinocytes of intact epidermis, as well as their expression in primary keratinocytes induced to terminally differentiate by elevated levels of extracellular calcium. Collectively, our results suggest a role for C/EBP isoforms in the regulation of genes involved in or specifically expressed during the process of squamous differentiation in epidermis.

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