GENERAL ABSTRACT

SHIM, MINSUB. Proteasomal Regulation of CCAAT/Enhancer Binding Protein alpha (C/EBPα) and Diminished Expression of C/EBPα in Squamous Cell Carcinomas

The CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors. C/EBPα is involved in mitotic growth arrest/differentiation and has been implicated as a human tumor suppressor in acute myeloid leukemia. C/EBPα is abundantly expressed in mouse keratinocytes. The purpose of this study was to examine the regulation of the C/EBPα protein and to determine if C/EBPα expression/function is altered in skin squamous cell carcinomas.

We found that C/EBPα is a short-lived protein with a half-life of ~ 1 hour and treatment with proteasome inhibitors blocked the degradation of C/EBPα protein. Poly-ubiquitinated C/EBPα were detected in BALB/MK2 and C/EBPα was degraded by the proteasome in an ATP- and ubiquitin-dependent manner. GSK3 is a known C/EBPα kinase and treatment of keratinocytes with LiCl, an inhibitor of GSK3 resulted in; i) an increase in C/EBPα protein levels, ii) increased electrophoretic mobility of C/EBPα protein and iii) no increase in C/EBPα mRNA levels suggesting that GSK3-mediated phosphorylation of C/EBPα may target it for proteasomal degradation. However, a mutant C/EBPα containing mutations in the GSK3 phosphorylation sites (T222A and T226A) retained its response to LiCl and additional pharmacological inhibitors of GSK3 did not alter C/EBPα levels indicating the effects of LiCl on C/EBPα are GSK3-independent. LiCl treatment inhibited C/EBPα degradation and

produced a six-fold increase in the half-life of C/EBPα protein. In vitro studies revealed that LiCl inhibited proteasomal degradation of C/EBPα. These results demonstrate C/EBPα is degraded via a proteasomal pathway and LiCl stabilizes C/EBPα through a GSK3 independent pathway involving inhibition of proteasome activity.

The expression of C/EBPα was evaluated in mouse skin SCC lines. C/EBPα mRNA and protein levels were greatly diminished or undetectable in all seven SCC cell lines compared to normal keratinocytes. Forced expression of C/EBPα resulted in the inhibition in SCC cell proliferation. Expression of C/EBPα also resulted in the expression of loricrin, a late stage marker of squamous differentiation. Treatment with 5'-aza-deoxycytidine increased C/EBPα expression in some SCC cell lines suggesting the C/EBPα promoter region may be transcriptionally silenced by hypermethylation. C/EBPα expression was negligible in all 14/14 SCC examined compared to normal epidermis. These results suggest the loss of C/EBPα expression may contribute to the altered growth and differentiation characteristics of skin SCCs.

PROTEASOMAL REGULATION OF CCAAT/ENHANCER BINDING PROTEIN ALPHA (C/EBPα) AND DIMINISHED EXPRESSION OF C/EBPα IN SQUAMOUS CELL CARCINOMAS

by

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BIOGRAPHY

Minsub Shim was born on January 21, 1967 in Seoul, The Republic of Korea. He enrolled Yonsei University, Seoul, The Republic of Korea in 1986 where he earned a Bachelor of Science degree in Biochemistry in 1990. He attended the Graduate School of Yonsei University in 1991 and got a Master of Science degree in Biochemistry in 1993. He then began working as a researcher in LG R&D Institute, Daejeon, The Republic of Korea until 1998. And then he started working toward a Ph.D. in Toxicology at North Carolina State University, Raleigh, North Carolina under the direction of Dr. Robert C. Smart in the summer of 1998.

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GENERAL INTRODUCTION

The regulation of gene expression is complex and is subject to multiple levels of control including the regulation of the rate of mRNA synthesis, the stability of the mRNA, the rate at which the mRNA is translated, as well as the stability of the translated product. The most common type of regulation is at the transcriptional level which controls the rate of mRNA synthesis. Every gene has sequences upstream of its coding sequence to control the rate of transcription. These sequences can be divided into two general classes; i) sequences which control basal transcription and ii) sequences which control the rate of transcription in response to cellular signals. At the transcriptional starting point, there is a tendency for the first base of mRNA to be adenine, flanked on either side by pyrimidines. This region is called the initiator (Inr) and most promoters have a sequence called the TATA box, usually located ~ 25 bp upstream of starting point. These sequences provide binding sites for general transcription machinery which includes RNA polymerase II, TATA binding proteins (TFIID, TFIIA, TFIIB, TFIIF, TFIIE, THIIH, and TFIIJ), and activators. While general transcription machinery initiates and maintains RNA transcription, specific transcription factors are responsible for the control of transcription in response to various stimuli. The sequences that these factors bind to are called response elements. There are ~2000 to 3000 transcription factors in mammals and it is these site-specific transcription factors that mediate the final steps of cell signaling from cell surface to nucleus. Transcription factors contain at least two domains ; i) a DNA binding domain that directly binds to gene-specific regulatory sites and ii) a transactivation domain that

functions to activate or suppress transcription.

CCAAT/ENHANCER BINDING PROTEIN (C/EBP)

CCAAT/Enhancer Binding Proteins are members of bZIP transcription factors

CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors. This class of transcription factor also includes Fos and Jun proteins which bind to AP-1 site, CREB/ATF (cAMP responsive element binding protein/activation transcription factor), PAR-domain proteins, and yeast/plant derived bZIP proteins (1). bZIP transcription factors are characterized by a basic DNA binding domain and a leucine-zipper region. The leucine zipper region is a heptad repeat of leucines within a 35 amino acid sequence which forms an amphipatic α-helix with the leucines being located on one side of the helix per every two turns (2). These leucines allow the two α-helices of separate monomers to dimerize by hydrophobic interactions (zipping). The first C/EBP protein, C/EBPα was identified as a heat-stable factor in rat liver nuclei and since it bound to the CCAAT box present in several promoters as well as to the core homology sequence [TGTGG(A/T)(A/T)(A/T)G] of certain viral enhancers, it was termed CCAAT/enhancer binding protein (3). Later, other members of C/EBP family were identified and all of C/EBP family members have conserved C-terminal parts, which is a basic amino acid rich DNA binding domain and a leucine zipper domain (4-10).

C/EBP Family Members and Tissue Expression Profile

The C/EBP family of transcription factors contains six members which are

C/EBPα (also known as C/EBP, RcC/EBP-1) (2, 8, 9, 11-14), C/EBPβ (also known as NF-IL6, IL-6DBP, LAP, CRP2, NF-M, AGP/EBP, ApC/EBP) (4-6, 8, 9, 14-18), C/EBPδ (also known as NF-IL6β, CRP3, CELF, RcC/EBP2) (8, 9, 13, 14, 19-21), C/EBPε (also known as CRP-1) (9, 12, 18, 22-24), C/EBPγ (also known as Ig/EBP-1) (7, 14) and C/EBPζ (also known as CHOP-10, GADD153) (10, 25, 26). The expression profile of each C/EBP member has been described at the mRNA and protein level in various tissues (4-11, 14, 23, 24, 27, 28). There are some discrepancies between the levels of C/EBP mRNA and protein (6, 9), suggesting the existence of post-transcriptional regulatory mechanisms. C/EBPα is highly expressed in liver, adipose, lung, peripheral leukocytes, epidermis, intestine and skeletal muscle (8, 9, 11, 28, 29). In liver, adipose, and epidermis, the highest level of C/EBPα mRNA expression was observed in terminally differentiated cells. C/EBPβ expression is highest in liver, intestine, lung, adipose, spleen, kidney, epidermis, and myelomonocytic cells (4, 8, 9, 15, 16, 27), while C/EBPδ is expressed in adipose, lung, and intestine (8, 9, 19). C/EBPγ and C/EBPζ are expressed ubiquitously (7, 10) and C/EBPε is highly expressed in myeloid and lymphoid cells (22-24, 30).

Structure and Properties of C/EBPs

As mentioned previously, all C/EBP family transcription factors have a highly conserved C-terminal bZIP motif, which includes a DNA binding domain that is rich in basic amino acids and a leucine zipper domain that mediates dimerization between C/EBP isoforms. C/EBP family members can either homodimerize or heterodimerize with other members of the C/EBP family through their dimerization domain (leucine zipper domain) (4-10, 27) and dimerization is a prerequisite for DNA binding (2).

Dimerization between different isoforms has the potential to increase the repertoire of transcriptional responses resulting from various stimuli. For example, a tissue that expresses the α and β isoforms will contain three species of C/EBPs : the α/α homodimer, the α/β heterodimer, and the β/β homodimer. In contrast to the C-temini, the N-termini of C/EBPs are less conserved (less than 20 % sequence homology). The N-termini of C/EBP family (except for C/EBPγ and C/EBPζ) contains three activation domains which are required for binding to general transcription factors and activating transcription (31-36). In addition, N-termini of C/EBPα, C/EBPβ, and C/EBPε contain negative regulatory domains which silence the function of activation domains (31, 33-35, 37, 38). While most of C/EBP transcription factors function as transcriptional activators, some C/EBP transcription factors function as negative regulators of transcription. For example, the N-terminally truncated form of C/EBPβ, LIP (liver inhibitory protein), lacks a transactivation domain and can bind to full-length C/EBPβ or other C/EBP transcription factors resulting in non-functional C/EBP homo- or heterodimers (39). C/EBPγ and C/EBPζ can also function as dominant negative inhibitors (10, 36). Recently, it has been shown that mutations occur in C/EBPα in certain forms of acute myeloid leukemia and these mutations result in a truncated form of C/EBPα that functions as a dominant-negative inhibitor of full-length C/EBPα (40). Protein-protein interactions between C/EBP transcription factors and other classes of protein molecules have been reported, such as C/EBPα-NFκB (41), C/EBPβ-NFκB (42-45), C/EBPδ-NFκB (46, 47), C/EBPβ-glucocorticoid receptor (48), C/EBPβ-Sp1 (49), C/EBPβ-Myb (50), and C/EBPα-AML1 (51) interactions. For C/EBPs interaction with the NFκB or glucocorticoid receptor, it has been shown that the leucine zipper domain in C/EBPs mediates this interaction (42, 45). In addition to

direct protein-protein interaction, the functional interaction of C/EBPs with p300 (52), Rb (53, 54), and p21 (55, 56) has been reported.

C/EBP family members have simple gene structures. C/EBPα, C/EBPβ, and C/EBPδ are intronless genes (6, 8, 9, 57) while the mouse C/EBPγ gene consists of two exons (58). C/EBPε gene has three exons and C/EBPζ has four exons (23, 25). The promoter of C/EBPα gene is subject to autoregulation which occurs in human and mouse by different mechanisms (59, 60). While C/EBPα binds to the murine C/EBPα promoter and results in an increased C/EBPα expression, human C/EBPα induces USF, another transcription factor, which induces C/EBPα expression. C/EBPα promoter can be repressed by nuclear factor C/EBP undifferentiated protein (CUP), an isoform of activator protein-2α (AP-2α), which binds to repressive elements in the C/EBPα gene promoter and silence the gene until late in the differentiation program (61). There are two isoforms of C/EBPα (42 kD and 30 kD) and the smaller isoform of C/EBPα (30 kD) can be generated by initiating translation at an alternative in-frame AUG codon (62, 63). This smaller isoforms still has an intact DNA binding domain and leucine-zipper domain however, its transactivating activity is low as it also has an altered transactivation domain. C/EBPβ mRNA can give rise to three C/EBPβ isoforms (38 kD, 35 kD, and 20 kD) among which 35 kD (LAP-liver activating protein) and 20 kD (LIP-liver inhibitory protein) are the major isoforms found in cells (39). While these forms of C/EBPβ are considered to result from alternative translation start sites, Baer et al showed that 20 kD C/EBPβ is generated predominantly by in vitro proteolytic cleavage during its isolation from cells (64). Four forms of C/EBPε (32 kD, 30 kD, 27 kD, and 14 kD) are encoded by alternative promoter usage and differential splicing (24).

Like LIP, C/EBPγ and C/EBPζ can function as negative regulators of C/EBP activity although their inhibitory mechanisms are different. While C/EBPγ lacks transactivation domain (36), C/EBPζ contains two proline residues in the basic region resulting in disruption of α-helical structure (10). C/EBPζ can heterodimerize with other C/EBP members however, such heterodimers are not able to bind to target sequences because of the altered DNA binding domain.

Biological Function of C/EBPs

The function of C/EBP family members has been investigated using target gene promoter analysis, overexpression/inhibition of C/EBPs, and gene-knockout animals. These analyses revealed roles of C/EBP family members in a number of cellular processes, including growth arrest/differentiation, inflammation, liver regeneration, metabolism, and other cellular signaling events.

(I) Role of C/EBPs in Cell Differentiation

It has been shown that C/EBP transcription factors have an important role in preadipocyte differentiation (65-67). The promoter region of many adipocyte-specific genes contains C/EBP binding sites and C/EBP transcription factors have a critical role in preadipocyte differentiation. C/EBPα expression is highest in terminally differentiated adipocytes (28) and the induction of C/EBPβ and C/EBPδ mRNA precedes C/EBPα mRNA expression during preadipocyte differentiation (8, 59, 67). Since C/EBPα promoter is activated by all three C/EBP isoforms (C/EBPα, C/EBPβ, and C/EBPδ), C/EBPβ and C/EBPδ have been proposed to function in early preadipocyte differentiation by inducing C/EBPα transcription, which in turn induces

transcription of adipocyte-specific genes. Overexpression of C/EBPα or C/EBPβ induced preadipocyte differentiation while inhibition of C/EBPα by antisense RNA blocked preadipocyte differentiation (68-71). Furthermore, embryonic fibroblasts from C/EBPβ and C/EBPδ deficient mice were unable to differentiate in response to hormonal stimulus and C/EBPα deficient mice had dramatically reduced lipid accumulation in adipose tissue (72, 73). C/EBP transcription factors also have important roles in differentiation of myeloid cells (74). The promoter region of many genes expressed in myeloid cells contains C/EBP binding sites and ectopic expression of C/EBPs activates a number of target genes in myeloid cells. Studies from C/EBPα knockout mice showed that these mice lack mature neutrophils (75). This defect correlates with a lack of receptors for granulocyte colony stimulating factor (G-CSF) or interleukin-6 (IL-6) and expression of these receptors restores granulopoiesis in C/EBPα deficient mice (75, 76). C/EBPε deficient mice generate atypical granulocytes that are functionally defective, lacking an oxidative burst (24). Lastly, C/EBPβ deficient mice show defects in macrophage activation, suggesting a potential role of C/EBPβ in macrophage activation or differentiation (77-79). In addition to adipocytes and myeloid cells, C/EBPs are implicated in differentiation of other type of cells including hepatocytes, mammary epithelial cells, keratinocytes, intestinal epithelial cells, and neuronal cells (28, 80-84). Studies with C/EBPβ deficient mice show that C/EBPβ is important for functional differentiation of mammary epithelial cells and expression of milk proteins. Female C/EBPβ deficient mice are sterile due to defects in differentiation of periovulatory granulosa cells in response to luteinizing hormone (85).

(II) Role of C/EBPs in Energy Metabolism

C/EBP transcription factors are also important in the control of energy metabolism. C/EBPα deficient mice die within a few hours due to hypoglycemia. This hypoglycemia results from two metabolic defects in liver. i) the level of liver glycogen is undetectable because of the reduced expression of glycogen synthase. ii) the expression of glucose-6-phosphatase, phosphoenolpyruvate carboxykinase, and tyrosine aminotransferase, which are required to synthesize glucose, are decreased (73). In initial studies on C/EBPβ deficient mice, it was observed that C/EBPβ deficient mice were generally healthy (78, 79), however, a second study by Croniger et al (86) showed that mice with A-phenotype survived, whereas mice with B-phenotype died soon after birth due to hypoglycemia, similar to C/EBPα deficient mice. While C/EBPα deficient mice lack liver glycogen, C/EBPβ deficient mice with B-phenotype are able to synthesize/store glycogen but unable to mobilize hepatic glycogen (87). The basis for the different phenotypes of the C/EBPβ deficient mice has not been described, however, some evidence suggests that the expression of non-inbred modifier genes are involved (87).

(III) Role of C/EBPs in Inflammation

The activity and expression levels of C/EBP transcription factors are regulated by inflammatory stimuli and different cytokines. For example, C/EBPβ and C/EBPδ mRNA are highly upregulated by lipopolysaccharide (LPS) and cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (77, 88-90). C/EBPβ was originally identified as an inducible protein by IL-6 or IL-1 (4, 5). The regulatory region of genes which encode cytokines/cytokine receptors, acute-phase

plasma proteins in hepatocytes, and components of inflammatory signaling pathways have binding sites for C/EBPs and gene knockout studies have provided further evidence that C/EBP transcription factors are important in regulation of the inflammatory responses (74, 77). Acute phase proteins are plasma proteins whose levels are regulated during the acute phase of inflammation (91). These liver-specific or liver-enriched proteins can be divided into two classes. Class I genes require both interleukin-1 (IL-1) and IL-6 for maximal induction, whereas class II genes are responsive to IL-6. It has been shown that the promoter regions of most class I genes (hemopexin, haptoglobin, α1-acid glycoprotein, serum amyloid A1, A2, and A3, complement C3, C-reactive protein) contain C/EBP binding motifs (77). C/EBPβ deficient mice show impaired expression of some acute phase genes (serum amyloid A and P proteins, C3) and TNF-α while the expression of other acute phase genes (hemopexin, haptoglobin) are not affected, possibly due to compensation by other C/EBP members (77). C/EBP binding motifs are also present in regulatory region of genes encoding several cytokines such as IL-6, IL-1β, TNF-α, IL-8, IL-12 and proteins which are important in macrophage/granulocyte functions such as nitirc oxide synthase, lysozyme, myeloperoxidase, granulocyte colony stimulating factor (74). Serum IL-6 levels are not changed in C/EBPβ deficient mice however serum IL-6 levels are elevated in aging C/EBPβ deficient mice (79, 92), suggesting C/EBPβ is dispensable for IL-6 gene expression. However, C/EBPβ regulates TNF-α and serum TNF-α induction is impaired in C/EBPβ deficient mice (79). In addition, deletion of C/EBPε gene, a C/EBP family member expressed in cells of the myelomonocytic lineage, resulted in decreased expression of several cytokines such as interferon-γ (IFN-γ), TNF-α, IL-2, IL-4, and IL-12 in the spleen (24).

(IV) Role of C/EBPs in Cell Proliferation

C/EBPα negatively regulates cell proliferation. C/EBPα expression is highest in terminally differentiated adipocytes/hepatocytes/keratinocytes while C/EBPα expression is down-regulated after partial hepatectomy (proliferation) (28). Overexpression of C/EBPα results in strong growth arrest in cultured cells (93) and abnormal cell proliferation is observed in liver and lung of C/EBPα deficient mice (94). Furthermore, the expression levels of C/EBPα are reduced in some tumors/tumor cell lines (95, 96). However, the importance of C/EBPα in the regulation of growth arrest and differentiation is exemplified by recent studies in which dominant-negative mutations in C/EBPα were found in some patients with human acute myeloid leukemia (AML) (40). Such mutations in C/EBPα are thought to result in a differentiation block of the granulocytic blasts and have implicated C/EBPα as a tumor suppressor gene. In addition, it has been suggested that AML1-ETO, a fusion protein resulting from t(8;21) translocation in AML, suppresses C/EBPα expression indirectly by inhibiting positive autoregulation of the C/EBPα promoter (97). The growth and differentiation regulatory functions of C/EBPα are complex and multifaceted. For example, C/EBPα has been proposed to regulate p21 expression (56) and interact with Rb family proteins (98). C/EBPα also has been shown to directly repress E2F function through its physical associations with E2F and this repression is necessary for growth arrest and adipocyte and granulocyte differentiation (99). However, recent studies indicate that C/EBPα can block growth independent of its DNA binding and transcriptional activity by forming a complex with cdk2 (cyclin-dependent kinase 2) and cdk4 and thereby blocking cyclin-cdk interactions and cell cycle progression (100). In addition, Harris

et al showed that C/EBPα cooperates with p21 to inhibit cdk2 activity (101). Thus, in addition to its DNA binding/transcription factor activity, C/EBPα can modulate growth arrest and differentiation by protein/protein interactions with cell cycle regulatory proteins independent of its transcription activity.

Regulation of C/EBPs

(I) Transcriptional/Translational Control

The levels and activity of C/EBPs are modulated by various stimuli, such as cytokines, hormones, nutrients, and cellular stresses. The levels and activity of C/EBPs are subjected to regulation at the transcriptional, translational, and post-translational level. In addition, the degradation of C/EBPs can affect the levels of C/EBPs in the cell. A number of signals can negatively or positively affect C/EBP mRNA levels (74) and the promoter regions of C/EBPα, β, δ, ε, ζ have been investigated. The C/EBPα promoter region has binding sites for C/EBP, Sp1, nuclear factor (NF)-1, NF-Y, c-myc, and upstream stimulating factor (USF) and is activated by overexpression of C/EBPα or C/EBPβ (59, 102), indicating C/EBPα promoter can be autoactivated. Autoactivation of C/EBPβ and C/EBPδ promoter also has been observed in some species (21, 103). Interestingly, c-myc, a proto-oncogene, inhibits transcription of C/EBPα by binding the core promoter region of C/EBPα (104, 105). The promoter of C/EBPε is transcribed by two alternative promoters which contain no TATAAA box but has a number of purine rich sequences for the Ets family of transcription regulators (23, 24). The C/EBPε promoter also has a retinoic acid response element (106). C/EBPζ mRNA is induced by growth arrest/DNA

damage/cellular stress (26) and the C/EBPζ promoter contains an AP-1 binding site which is responsible for C/EBPζ induction by oxidative stress (107). The C/EBPζ promoter has a conserved C/EBP site which has an important role in its induction during acute phase response in hepatocytes (108). Production of different C/EBPα or C/EBPβ isoforms by usage of alternative translation initiation codons is a major mechanism of translational control (12, 39, 62, 63).

(II) Post-Translational Control

Transactivation, DNA binding activity, or localization of C/EBPs can be modulated by post-translational modification. Phosphorylation is the most frequent post-translational modification and several kinases that phosphorylate C/EBPs have been identified. The transactivation activity of C/EBPβ can be induced by phosphorylation of Thr235 by a Ras/mitogen-activated protein kinase (MAPK) pathway (80, 109), Ser105 by protein kinase C (PKC) (110), and Ser 276 by Ca^{2+}/calmodulin-dependent protein kinase (111). TGF-α induces activation of the p90 ribosomal S kinase (RSK), which results in the phosphorylation of rat C/EBPβ on Ser-105 and of mouse C/EBPβ on Thr-217 (112). C/EBPζ is phosphorylated by p38 MAPK during cellular stress and this phosphorylation results in increased transactivation activity (113). PKC-mediated phosphoryation of C/EBPα at Ser248, Ser277, and Ser299 attenuates its DNA binding activity (114) and insulin induced phosphorylation of C/EBPβ by PI3 kinase (phosphatidylinositol-3-kinase) suppresses transactivation activity of C/EBPβ (115). DNA binding activity of C/EBPβ is negatively regulated via phosphorylation of Ser240 by PKC and in vitro phosphorylation study showed that protein kinase A (PKA) mediated phosphorylation at Ser173, Ser233, and Ser240

inhibits DNA binding activity (116). On the other hand, forskolin-induced activation of cAMP/PKA in rat PC12 cells results in the translocation of C/EBPβ into the nucleus, where it activates c-fos gene transcription (117). Antioxidant-induced nuclear translocation of C/EBPβ in colorectal cancer cell line is also regulated by PKA mediated phosphorylation (118). Glycogen synthase kinase 3 (GSK3) has been identified as a C/EBPα kinase (119). However, the functional significance of this phosphorylation is not known. Transcriptional activity of C/EBPε as well as other C/EBP family members (C/EBPα, C/EBPβ, and C/EBPδ) is positively controlled by sumoylation at a conserved inhibitory domain (120). Subramanian et al showed that C/EBPα interacts directly with the E2 SUMO-conjugating enzyme Ubc9 and can be sumoylated in vitro (121). Recently, it has been shown that C/EBPγ and C/EBPζ are ubiquitinated and subsequently degraded by the proteasome (122).

CCAAT ENHANCER BINDING PROTEINS IN EPIDERMIS

Epidermis

The epidermis is a stratified squamous epithelium composed mainly of keratinocytes that form four different layers. In addition to retaining body fluids to prevent dehydration, the epidermis provides an essential barrier function against environmental insults such as physical/chemical trauma and microorganisms. Each epidermal layer represents a different stage in epidermal differentiation beginning from basal keratinocytes which become post-mitotic and move upwards through the spinous and granular layers, resulting in non-viable stratum corneum. A subset of basal

epidermal cells is basal stem cells which generate transient amplifying cells that undergo a limited number of divisions and withdraw from cell cycle. As these post-mitotic cells move upward, epidermal differentiation begins and when they reach the outer surface, they become enucleated, flattened, and dead cells that are subsequently sloughed and replaced by inner cells that move upwards (123). Therefore, the epidermis is a tissue which is in a constant state of dynamic equilibrium, replenishing itself throughout life.

During epidermal differentiation, the expression and the repression of specific genes are strictly regulated as a basal keratyinocyte transits through the different stages of differentiation (124). For example, the transition of basal keratinocytes from the basal layer to the spinous layer is accompanied by the repression of keratin 5 and keratin 14 and upregulation of keratin 1 (K1) and keratin 10 (K10) which are early differentiation markers. As a keratinocyte moves from spinous to granular layer, the expression of K1 and K10 is suppressed while the expression of late differentiation markers, involucrin and loricirn, are induced. Finally, epidermal transglutaminase cross-links these structural proteins and forms a cornified envelope, resulting in the mature squame following digestion of intracellular organelles.

Role of C/EBPα and C/EBPβ in epidermal differentiation

The expression of C/EBP family members in mouse and human epidermis has been investigated and some of C/EBP family members are highly expressed in epidermis (29, 125, 126). C/EBPα, C/EBPβ, and C/EBPδ mRNA are expressed abundantly in mouse epidermis and the levels of C/EBPα, C/EBPβ, and C/EBPδ mRNA

expression in the epidermis are higher in epidermis than in liver and in lung, tissues known to express high levels of these C/EBP members (29). C/EBPα and C/EBPβ proteins are expressed abundantly in mouse keratinocytes while C/EBPδ protein expression is low. C/EBPα protein is expressed in the nuclei and cytoplasm of suprabasal kertinocytes and is weakly expressed in a perinuclear manner in some basal keratinocytes. C/EBPα expression is observed in non-proliferative compartments of hyperplastic epidermis and papillomas however, C/EBPα expression is greatly diminished in squamous cell carcinomas (SCC). C/EBPβ expression shows a highly ordered and specific pattern in epidermis. C/EBPβ expression is exclusive to the nuclei of three-cell clusters of suprabasal keratinocytes and these clusters are repeated at regular intervals throughout the epidermis, which is consistent with the central suprabasal column of the epidermal proliferative unit.

It has been shown that C/EBPα and C/EBPβ are associated with epidermal differentiation. The terminal differentiation of keratinocytes can be induced by increasing the level of extracellular calcium in the media (127). When terminal differentiation is induced by calcium, expression of C/EBPα and C/EBPβ is increased (29). In addition, forced expression of C/EBPα or C/EBPβ induces differentiation-like morphological changes and growth arrest in BALB/MK2 keratinocytes. C/EBPβ has been shown to modulate keratin 1 (K1) and keratin 10 (K10) expression, early keratinocyte differentiation markers. Forced expression of C/EBPβ increases K1 and K10 expression in BALB/MK2 keratinocytes (80) and the promoter region of the K10 gene contains three functional C/EBP binding sites (128). Primary keratinocytes isolated from C/EBPβ deficient mice display decrease in K1 and K10 expression and

are resistant to calcium induced growth arrest (80). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is a potent inducer of keratinocyte differentiation and of involucrin gene expression (129), a late keratinocyte differentiation marker. Agarwal et al showed that a C/EBP site in the proximal regulatory region of the human involucrin gene is required for involucrin expression in response to phorbol ester (130). The human involucrin reporter plasmid is activated by C/EBPα whereas C/EBPβ and C/EBPδ inhibit both TPA- and C/EBPα-dependent involucrin promoter activation. In addition, a C/EBP binding site has been identified in the promoter region of α2 and α5 integrin genes in human keratinocytes and the overexpression of C/EBPα or C/EBPβ inhibited the activity of these promoters (131). Since detachment of keratinocytes from basement membrane triggers terminal differentiation and integrin expression is down-regulated during keratinocyte terminal differentiation, the up-regulation of C/EBPs may contribute to the inhibition of integrin expression during keratinocyte differentiation.

Role of C/EBPs in Epidermal Proliferation and Cell Survival

C/EBPs also have an important role in epidermal proliferation and cell survival. It has been shown recently that C/EBPβ deficient mice are completely refractory to a variety of carcinogen-induced skin tumors and H-ras transgenic mice that carry the C/EBPβ deletion mutation display a significant reduction in tumorigenesis (132). In addition, oncogenic ras potently stimulated C/EBPβ transactivation activity and C/EBPβ cooperated with ras to transform NIH 3T3 cells, suggesting C/EBPβ as a downstream mediator of ras signaling. Ras-induced C/EBPβ transactivation activity is abolished when Thr188, an extracellular signal regulated kinase (ERK) 1/2

phosphorylation site, is substituted with alanine. The positive role of C/EBPβ in cell proliferation also has been described in other systems. For example, Greenbaum et al showed that C/EBPβ mediates growth response pathways in the regenerating liver and is required for a normal proliferative response (133) and Buck et al showed that p90 ribosomal S kinase (RSK) mediated phosphorylation of rat C/EBPβ on Ser-105 and of mouse C/EBPβ on Thr-217 is critical for TGF-α induced proliferation of hepatocytes (112). Recently, it was reported that a certain C/EBPβ isoform transforms normal mammary epithelial cells and induces an epithelial to mesenchymal transition in culture (134).

C/EBPβ also appears to be important in regulating cell survival. For example, a seventeen-fold increase in the number of apoptotic keratinocytes is observed in DMBA-treated, C/EBPβ deficient epidermis compared with wild-type epidermis (132). The observed increase in the number of apoptotic cells in DMBA-treated, C/EBPβ deficient epidermis is consistent with a survival/anti-apoptotic role for C/EBPβ. It is possible that in normal keratinocytes C/EBPβ regulates epidermal differentiation as well as survival, which is required to complete the differentiation program. However, in the presence of oncogenic H-ras, the prosurvival function of C/EBPβ may dominate over the differentiating function of C/EBPβ and C/EBPβ may contribute to clonal expansion of oncogenic H-ras containing cells. It has been proposed that in C/EBPβ deficient epidermis the prosurvival function of C/EBPβ is blocked and cells containing oncogenic ras mutations undergo apoptosis, inhibiting tumor formation. In support of this, Buck et al observed that hepatotoxin CCl_4 activates RSK, resulting in the phosphorylation of Thr217 in mouse C/EBPβ and induces proliferation of stellate cells, whereas CCl_4

treatment induces apoptosis in C/EBPβ deficient mice (135). They showed that C/EBPβ phosphorylation on Thr-217 by RSK creates a functional XEXD caspase substrate/inhibitor box and inhibits activation of procaspase 1 and 8.

UBIQUITIN/PROTEASOME-MEDIATED PROTEOLYTIC SYSTEM

The cellular integrity is maintained by dynamic equilibrium between synthesis and degradation of each protein. Cells must control the rates of both synthesis and destruction of their proteins in order for proper cellular function. For proper degradation of target proteins, cells contain two major degradation machineries: i) proteases (cathepsin B, D, H, and L, calpain I and II) and ii) multicatalytic, multisubunit ubquitin/proteasome in nucleus/cytoplasm. While proteases make several cuts in its target protein without energy input resulting in several fragments of polypeptide, ubiquitin/proteasome system requires energy expenditure and cleaves every peptide bond in target protein. Recently, many cellular proteins involved in the critical cellular processes, such as proliferation, differentiation, DNA repair, and apoptosis, have been discovered to be degraded by the ubiquitin/proteasome pathway (136, 137). Furthermore, deregulated proteasomal degradation has been found in a number of human diseases, including cancer, neurodegenerative diseases, and myodegenerative diseases and proteasome inhibitors are being developed as a therapeutic agent.

Structure of Proteasome

The core proteasome consists of the 20S core unit (~ 700 kD) and the structure

of the 20S proteasome is well conserved in all organisms from archaebacteria to yeast to human (138-140). The 20S core unit forms the hollow barrel shape in which four heptameric rings are stacked forming central chamber where proteolysis occurs. Two outer rings are composed of seven α-subunits and two inner rings are composed of seven β-subunits. Eukaryotic proteasomes have two additional 19S regulatory units (141) which bind to the 20S proteasome forming a lid and a base in the 26S proteasome. 19S units contain binding sites for ubiquitinated proteins, enzymes that depolymerize ubiquitin chain, and six distinct ATPases. The ATPase function of the 19S unit unfolds target protein and facilitates the entry of target protein into the 20S proteasome (142). To ensure that every peptide bond in target protein is susceptible for cleavage, 20S proteasome has multiple proteolytic activities: i) chymotrypsin-like, ii) trypsin-like, iii) caspase-like, iv) branched-amino acid preferring, and v) small neutral amino acid-preferring (143-145). Based on mutational studies in yeast, these distinct proteolytic activities are mapped to different subunits in 20S proteasome (146-150).

Ubiquitin/Proteasome System

In ubiquitin/proteasome system, the degradation of a protein begins with the conjugation of multiple ubiquitins to the target protein (151) although certain proteins do not require ubiquitination for proteasomal degradation (152). Ubiquitin is a well-conserved, ubiquitously expressed small molecular weight (76 amino acids) protein. Ubiquitin is conjugated to a target protein through a reversible isopeptide bond catalyzed by a sequential action of E1, E2, and E3 ligase. The conjugation reaction is initiated by formation of a high-energy thioester bond between the C-terminus of ubiquitin (Gly76) and the Cys of E1 ligase in an ATP dependent manner. The activated

ubiquitin is transferred to E2 ligase which transfers ubiquitin to E3 ligase. E3 ligase catalyzes the last step of the ubiquitin conjugation reaction and ubiquitin is ligated to ε-amino group of lysine residues in the target protein through an isopeptide bond. Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and each of these lysine residues can form an isopeptide bond with C-terminal glycine of next ubiquitin, resulting in polyubiquitination (153-155). However, the most common type of ubiquitin chain is the K-48 linked type chain which signals the target protein for proteasomal degradation (156). Most organisms contain one or two E1 ligases and it is these E1 ligases that catalyze activation of ubiquitin. A single E1 ligase is known in yeast (Uba1) and deletion of this gene is lethal in yeast (157). Many E2 ligases have been identified in yeast (13 E2 ligases) and mammals (more than 20 E2 ligases) (158, 159). Some E2 ligases are involved in specific cellular processes as ubiquitin conjugating enzymes (UBC). For example, yeast E2 ligase UBC2/RAD6 is involved in the degradation of N-end rule substrates and in DNA repair (160). UBC3/CDC34 is required for G1/S transition via the degradation of cell cycle regulators (160, 161). However, it is believed that specificity of E2 ligases in cellular processes results from their association with a distinct E3 ligase. E3 ligases are responsible for ligation of ubiquitin to the target protein for proteasomal degradation. They recognize specific motifs in their substrate and confer the substrate specificity. Four families of E3 ligase have been identified. i) N-end rule family, ii) HECT (Homologous to E6-AP carboxy terminus) domain family, iii) ring finger family, and iv) multisubunit complex family. E3α ligase (UBR1-yeast homologue of E3α) is a large protein (~ 200 kD) and contains three distinct functional domains for substrate recognition. A type-1 site recognizes basic residues (Arg, Lys, and His) in N-end rule substrates whereas a type-2 site

recognizes bulky hydrophobic residues (Leu, Ile, Phe, Trp, and Tyr) (162, 163). The third substrate binding site recognizes a non-N-end rule substrate (164, 165). The HECT family E3 ligase contains a 350 amino acid-long C-terminal sequence which is homologous to E6-AP (human papilloma virus E6 oncoprotein associating protein) and at least 20 members of this E3 ligase family have been identified (166, 167). E6-AP mediates the ubiquitination of p53, resulting in proteasomal degradation of p53. The ring finger motif was originally identified in human RING1 gene product and it contains the Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(2-3)}$-Y-X_2-Cys-$X_{(4-48)}$-Cys-X_2-Cys sequence, where X can be any amino acid and Y can be Cys or His (168). Proto-oncoprotein c-cbl is the prototype of ring E3 ligase which ubiquitinates receptor protein tyrosine kinase and targets it for proteasomal degradation (169-171). APC (anaphase-promoting complex) and SCF complex (Skp1, cullin/cdc53, and F-box proteins) are the member of the multisubunit E3 ligase complexes (172, 173). Known substrates for APC E3 ligase are certain cyclins, anaphase inhibitors, and spindle-associated proteins (174). SCF E3 ligase complex recognizes a number of important cell cycle regulators such as G1 cyclins, CDK inhibitor, and other short-lived proteins (173). It has been proposed that Skp1 binds to F-box proteins, a different component of the SCF complex, and the F-box proteins recognize substrates in a phosphorylation dependent manner (175-177). Isopeptide bond between ubiquitin and substrate can be cleaved by deubiquitinating enzymes such as UCH (ubiquitin C-terminal hydrolases) and UBP (ubiquitin-specific processing enzymes) (178). These enzymes are involved in i) regeneration of C-terminus of ubiquitin after degradation of attached protein, ii) reversing the ubiquitination from wrong target proteins, and iii) disassembly of polyubiquitin chains to replenish cellular ubiquitin pools.

The substrate proteins for ubiquitination are recognized by distinct ubiquitin ligases via specific motifs. Several structural motifs for ubiquitination have been identified including genetically programmed signal, phosphorylation, and damaged protein. Certain proteins contain short N-terminal sequences of amino acids and the nature of these N-terminal amino acids determines the rate of ubiquitination. This is the first identified structural motif for ubiquitination and is referred to as the N-end rule. Ubiquitination of target proteins can be positively or negatively regulated by phosphorylation. These target proteins include cyclins/cyclin dependent kinase inhibitors, checkpoint regulators, cellular signaling components (e.g. SMADs, TGF-β, IκBα, β-catenin, c-Jun, and p53), and cell surface receptors. Phosphorylated target proteins are recognized by F-box protein which is a component of SCF complex E3 ligase. Furthermore, the activity of ubiquitin ligase is regulated by phosphorylation. The exposure of hydrophobic residues which are normally embedded in protein-protein interface or inside the protein can function as a signal for ubiquitination. Exposure of such residues can result from protein misfolding or inappropriate assembly of the subunit into a multisubunit complex and proteasomal surveillance ensures the degradation of these unwanted proteins.

Proteasomal Defects Associated with Human Diseases

Since the proteasome regulates the cellular levels of numerous proteins, defects in the proteasomal system can result in human diseases. For example, a defect in the gene encoding human E3 ligase (UBE3A) is identified in Angelman's syndrome, a neurological disorder characterized by developmental delay, speech impairment, and

movement or balance disorder (179, 180). Another type of inherited proteasomal defect is Liddle syndrome which is characterized by abnormally high blood pressure due to incapability to maintain the balance of salt and water. In these patients, increased half-life of sodium channel due to deletion or mutation of ubiquitination site in sodium channel protein allows excessive reabsorption of sodium and water (181). Skeletal muscle wasting in some pathological states also results from accelerated ubiquitin-mediated proteolysis. US2 and US11, the proteins encoded by human cytomegalovirus (CMV), target MHC class I heavy chain molecule for degradation by directing it from the ER to the cytoplasm, thereby evading immune system (182). Accumulation of ubiquitin conjugates is observed in senile plaques, lysosomes, endosomes, and intracellular inclusion bodies in neurological disorders such as Alzheimer's disease and Parkinson's disease although it is not known whether this accumulation is a direct result of defects in the proteasomal pathway (183).

Most cancers are caused by activating mutations in proto-oncogenes and/or inactivating mutations in tumor suppressor genes and the proteasomal degradation system regulates the levels of many of these gene products (136)). Certain modifications during tumorigenesis can change the stability of these oncoproteins/tumor suppressor proteins by affecting the proteasome-mediated degradation and some proteins involved in tumorigenesis contain ubiquitin ligase activity. For example, p53 is targeted for proteasomal degradation by mdm2 which is a p53 target gene containing E3 ubiquitin ligase activity. While mdm2 targets p53 for degradation, mdm2 is self-ubiqutinated and degraded. Cyclin dependent kinase inhibitor p21waf/cip, another p53 target gene, is degraded by proteasome and GSK3 (glycogen synthase kinase 3)

mediated phosphorylation of p21waf/cip triggers its degradation (184). Rb (Retinoblastoma) protein is a tumor suppressor and negatively regulates G1/S transition by interacting with E2F transcription factor. Rb protein is degraded in ubiquitin dependent manner (185). In addition, free E2F is degraded in ubiquitin dependent manner by the 26S proteasome (186-188). Mutations in the tumor suppressor gene APC is associated with the development of colon cancer. Mutations in the APC gene leads to accumulation of β-catenin which is normally degraded via the proteasome and increased level of β-catenin is related to carcinogenesis (189, 190). Abl is a cellular homologue of the transforming gene of Abelson murine leukemia virus and activation of abl occurs as a consequence of a chromosomal translocation which generates a bcr-abl oncogenic fusion protein (191-194). It has been reported that bcr-abl fusion protein targets abl-interactor proteins (ABI1 and ABI2), which are inhibitors of tyrosine kinase activity of abl, for proteasomal degradation (195). Oncogenic protein myc has a very short half-life (30 min) and is degraded by the proteasome. Sears et al showed that ERK mediated phosphorylation of Ser62 is required for ras-induced stabilization of myc while GSK3 mediated phosphorylation of Thr58, which is dependent on the prior phosphorylation of Ser 62, is associated with the degradation of myc (196). Increased activity of the NFκB transcription factor promotes cell survival in some tumors (197-200) and NFκB is activated by the ubiquitin/proteasome pathway (201, 202). In response to stimuli, IκBα which normally complexes with NFκB is phosphorylated by IκB kinase and is ubiquitinated by SCF ubiquitin ligase for proteasomal degradation.

THESIS RESEARCH FOCUS AND HYPOTHESES

In manuscript I, we hypothesized that C/EBPα is degraded via proteasomal pathway and that GSK3 regulates this process by targeting C/EBPα for proteasomal degradation. GSK3 is a ser/thr kinase that phosphorylates and inhibits the activity of glycogen synthase and two highly homologous forms of mammalian GSK3, GSK3α and GSK3β, have been identified. GSK3β is expressed ubiquitously in eukaryotes including yeast, *C. elegans*, *Drosophila*, *Xenopus*, mice and humans and is expressed in most tissues (203). Recent studies have indicated that GSK3β phosphorylates a variety of cell cycle regulatory proteins including p21 (184), β-catenin (204), cyclin D (205) and c-myc (196) and in doing so targets these molecules for proteasomal degradation. We investigated the degradation mechanism of C/EBPα and demonstrated that C/EBPα is degraded via the ubiquitin/ATP-dependent proteasomal pathway and lithium, a known inhibitor of GSK3 (206, 207), inhibits C/EBPα degradation by interfering with the proteasomal degradation of C/EBPα in a GSK3-independent manner.

In manuscript II, we hypothesized that loss of C/EBPα is important in development of SCC. The role of C/EBPα as a negative regulator of cell proliferation has been suggested from the several studies and C/EBPα deficient mice display abnormal proliferation of certain type of cells (94). In addition, it has been reported that C/EBPα levels are reduced in hepatocellular carcinomas (208) and lung cancer cell lines (96). Recently, dominant-negative mutation in C/EBPα has been found in human acute myeloid leukemia and C/EBPα has been implicated as human tumor suppressor

gene (40). We demonstrated that C/EBPα expression levels are greatly diminished in mouse SCC / SCC cell lines and forced expression of C/EBPα inhibits the proliferation of SCC cell lines. In addition, we showed that C/EBPα is related to the differentiation of keratinocytes and transcription of C/EBPα gene may be silenced by promoter hypermethylation in SCC cell lines. Our results suggest a tumor suppressor function for C/EBPα in SCC development.

MANUSCRIPT I

Lithium Stabilizes C/EBPα Protein through a GSK3-Independent Pathway Involving Direct Inhibition of Proteasomal Activity

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Abbreviations; C/EBP : CCAAT/enhancer binding protein, GSK3 : Glycogen synthase kinase 3

ABSTRACT

C/EBPα, a basic leucine zipper transcription factor, is involved in mitotic growth arrest and differentiation. Given that numerous proteins involved in cell cycle regulation are degraded via the ubiquitin-proteasome system, we examined whether C/EBPα protein is degraded via a proteasomal mechanism. In cycloheximide-treated BALB/MK2 keratinocytes we found that C/EBPα is a short-lived protein with a half-life of ~ 1 hour. Treatment with proteasome inhibitors, MG-132 or lactacystin, blocked the degradation of C/EBPα protein. Higher molecular weight species of ubiquitinated C/EBPα were detected in BALB/MK2 and in vitro studies confirmed that C/EBPα is degraded by the proteasome in an ATP- and ubiquitin-dependent manner. GSK3 is a known C/EBPα kinase and treatment of keratinocytes with LiCl, an inhibitor of GSK3 resulted in; i) a five-fold increase in C/EBPα protein levels, ii) increased electrophoretic mobility of C/EBPα protein and iii) no increase in C/EBPα mRNA levels suggesting that GSK3-mediated phosphorylation of C/EBPα may target it for proteasomal degradation. However, a mutant C/EBPα containing T to A mutations in the GSK3 phosphorylation sites (T222A and T226A) retained its response to LiCl and additional pharmacological inhibitors of GSK3 did not alter C/EBPα levels indicating the effects of LiCl on C/EBPα are GSK3 independent. LiCl treatment of BALB/MK2 cells inhibited C/EBPα degradation and produced a six-fold increase in the half-life of C/EBPα protein. In vitro studies revealed that LiCl inhibited proteasome activity and the ensuing degradation of C/EBPα. These results demonstrate C/EBPα is degraded via an ubiquitin-dependent proteasomal pathway and LiCl stabilizes C/EBPα through a GSK3 independent pathway involving direct inhibition of proteasome activity.

INTRODUCTION

CCAAT/enhancer binding protein α (C/EBPα) is a member of the basic leucine zipper (bZIP) class of transcription factors. There are six members of the C/EBP family (C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, C/EBPγ and C/EBPζ) (1-3). The C-terminal region of C/EBP contains a basic domain that is responsible for binding specific DNA sequences and a leucine zipper domain that functions in dimerization (4). C/EBPα can form homodimers as well as heterodimers with other members of the C/EBP family. The N-terminal region of C/EBPα contains three transactivation elements and an additional highly conserved region (CR4) that is thought to have a regulatory function. C/EBPα is highly expressed in liver, fat, lung, peripheral leukocytes, epidermis, intestine and skeletal muscle (5-7). In a numerous cell types, including preadipocytes (8,9), various myeloid cells (10,11), hepatocytes (12) and keratinocytes (6,13), C/EBPα is involved in the regulation of mitotic growth arrest and/or differentiation. Consistent with this, C/EBPα null mice display cell proliferation defects in liver and lung (14,15). C/EBPα also plays a key role in energy homeostasis. C/EBPα null mice die shortly after birth due to altered hepatic glucose and glycogen metabolism and also display defects in white adipose tissue differentiation (14).

The importance of C/EBPα in the regulation of growth arrest and differentiation is exemplified by recent studies in which dominant-negative mutations in C/EBPα were found to be associated with human acute myeloid leukemia (16,17). Such mutations in C/EBPα are thought to result in differentiation block of the granulocytic blasts and have implicated C/EBPα as a tumor suppressor gene. The growth and differentiation regulatory functions of C/EBPα are complex and multifaceted. For example, C/EBPα has been proposed to regulate p21 expression (18,19) and interact with Rb family

proteins (20,21). C/EBPα has been shown to directly repress E2F function through its physical associations with E2F and this repression is necessary for growth arrest and adipocyte and granulocyte differentiation (22). However, recent studies indicate that C/EBPα can block growth independent of its DNA binding and transcriptional activity by forming a complex with cdk2 and cdk4 and thereby blocking cyclin-cdk interactions and cell cycle progression (23). Thus, it appears that in addition to its DNA binding/transcription factor activity, C/EBPα can modulate growth arrest and differentiation by protein/protein interactions with cell cycle regulatory proteins independent of its transcription activity. Therefore, the identification of cellular processes that impinge on the regulation of C/EBPα protein stability may be critical in understanding the regulation and/or deregulation of C/EBPα-induced growth arrest and differentiation.

The ubiquitin-proteasome degradation plays an important role in the degradation of cellular proteins which are involved in regulating various cellular process, including cell cycle regulation, differentiation and apoptosis (24,25). Recent studies have indicated that GSK3 phosphorylates a number of cell cycle regulatory proteins including p21 (26), β-catenin (27), cyclin D (28) and c-myc (29) and in doing so targets these molecules for proteasomal degradation. Lithium, an inhibitor of GSK3 (30,31), blocked the proteasomal degradation of these proteins. C/EBPα can be phosphorylated by GSK3 on Thr 222 and Thr 226 and this phosphorylation can be blocked by lithium (32,33). However, the functional significance of GSK3-mediated phosphorylation is not known nor is it known whether C/EBPα is degraded via a proteasomal pathway or whether GSK3 or lithium treatment can alter C/EBPα protein degradation/stability. While the effect of lithium on the inhibition of proteasomal

degradation of proteins that are GSK3 substrates has generally been attributed to its inhibition of GSK3, lithium also inhibits a number of other cellular kinases and more recently lithium has been shown to a be an inhibitor of chymotryptic activity of both the 20S and 26S proteasome (34). Therefore, we have examined whether C/EBPα is degraded via a proteasomal mechanism and investigated the role of GSK3 and lithium on C/EBPα protein stability. We demonstrate that C/EBPα is degraded via ubiquitin-dependent proteasomal pathway and that lithium stabilizes C/EBPα protein through a GSK3 independent pathway involving direct inhibition of proteasomal activity.

MATERIALS AND METHODS

Materials

Fetal bovine serum, trypsin, human recombinant epidermal growth factor (hEGF), Lipofectin, and tris-glycine gels were purchased from Invitrogen. Ca^{2+}-free Eagle's minimal essential medium (EMEM) was purchased from BioWhittaker. The enhanced chemiluminescence (ECL) reagents were purchased from PerkinElmer Life Sciences. HRP-linked donkey anti-rabbit IgG was purchased from Amersham. Ubiquitin, HeLa S-100, energy regeneration system (ERS),ubiquitin-aldehyde, MG-132 and lactacystin were purchased from Boston Biochem. Cycloheximide and monoclonal anti-Flag antibody were purchased from Sigma. Bio-Rad protein assay reagent was purchased from BioRad. Construction of pcDNA3-C/EBPα has been previously described (13). λ-phosphatase was purchased from New England Biolabs. GSK3 inhibitor SB216763 and SB415286 were from GlaxoSmithKline. pCMV-Flag expression vector was a kind gift from Dr. Jun-Tsuji (North Carolina State University, Raleigh). Rabbit polyclonal anti-ubiquitin antibody, rabbit polyclonal anti-C/EBPα antibody, rabbit polyclonal anti-p53 antibody, and protein A/G-plus agarose were purchased from SantaCruz Biotechnology.

Transfection and lithium treatment

BALB/MK2 keratinocytes (a gift from Dr. Weissman, University of North Carolina, Chapel Hill) were plated at 2.5 x 10^5 cells/60 mm culture dish in Ca^{2+} free EMEM supplemented with 8 % Chelex-treated fetal bovine serum, 4 ng of hEGF per ml, and 0.05 mM calcium chloride. Two days after plating, BALB/MK2 keratinocytes were transfecetd with 2 μg of pcDNA3-C/EBPα and 12 μg of Lipofectin 2 ml serum free EMEM containing 4 ng of hEGF per ml and 0.05 mM calcium chloride according to

manufacturer's protocol. Twenty-four hours following transfection, cells were refed with EMEM supplemented with 8 % Chelex-treated fetal bovine serum, 4 ng of hEGF per ml, and 0.05 mM calcium chloride and incubated with 20 mM lithium chloride for 24 hr.

Preparation of nuclear and whole cell lysates

Nuclear extracts were prepared as previously described by Schreiber et al (35). For the preparation of whole cell lysates, cells were washed with cold PBS, harvested by scraping, collected by brief centrifugation. Cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1mM PMSF, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 1mM sodium orthovanadate, 0.6 % NP-40) by sonication and then one-tenth volume of 5 M NaCl was added. Incubation mixture was vortexed, incubated for 15 min on ice, and centrifuged (14,000g, 10 min, 4 ^{o}C). Supernatants were stored at –80 ^{o}C until use. Protein concentration was determined by the Bio-Rad protein assay reagent.

Western blot analysis

Equal amounts of protein were precipitated by adding equal volume of 20% trichloroacetic acid and washed with acetone (-20 ^{o}C). Protein samples were solubilized and boiled in SDS sample buffer for 2 min, and then separated by SDS-PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (PBS with 1 % bovine serum albumin, 5 % non fat dry milk, and 0.1 % Tween-20) for 1 hr at room temperature, the membranes were probed for 2 hr at room temperature with rabbit polyclonal IgG raised against C/EBPα (SantaCruz Biotechnology). The membranes were washed and then probed with an HRP-linked secondary antibody for 1 hr at room temperature.

Detection was made with an enhanced chemiluminescence reagent followed by exposure of the membrane to film.

Luciferase assay

BALB/MK2 keratinocytes were plated at 1 x 10^5 cells/well in 12 well culture plate. Two days after plating, BALB/MK2 keratinocytes were transfecetd in triplicate with 100 ng of pcDNA3-C/EBPα and 400 ng of the specified C/EBP-dependent promoter/reporter plasmid as described in the text, and 3 μg of Lipofectin in 0.5 ml serum free EMEM according to manufacture's protocol. Forty-eight hours later, cells were harvested and the luciferase activity was determined by using luciferase assay kit (Promega). Protein concentration was determined with the Bio-Rad protein assay reagent.

Dephosphorylation of C/EBPα by phosphatase treatment

BALB/MK2 cells were transfected with pcDNA3-C/EBPα and 48 hr following transfection, nuclear extracts were isolated as described above, except that the phosphatase inhibitor (sodium orthovanadate) was omitted. 50 μg of nuclear extracts was incubated with 400 units of λ-phosphatase in 50 μl of phosphatase buffer (50 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, and 2 mM $MnCl_2$) at 30 °C for 1 hr. The reaction was stopped by adding equal volume of 20% trichloroacetic acid. Precipitated proteins were washed with acetone (-20 ^oC) and were solubilized and boiled in SDS sample buffer for 2 min. Protein samples were separated by SDS-PAGE and analyzed by western blotting.

Northern blot analysis

Total RNA was isolated from untransfected or pcDNA3-C/EBPα transfected BALB/MK2 cells using Promega's SV total RNA isolation kit. C/EBPα cDNA was

labeled with [α-32P] dCTP by using Ready-To-Go labeling beads (Amersham). RNA was electrophoresed on agarose gel containing formaldehyde, transferred to zeta-probe GT membrane (BioRad) and UV cross linked. Blots were incubated at 65 °C in hybridization buffer (0.25 M Na_2HPO_4 pH 7.2, 7 % SDS) and sequentially washed with washing buffer 1 (20 mM Na_2HPO_4 pH 7.2, 5 % SDS) and washing buffer 2 (20 mM Na_2HPO_4, 1% SDS) at room temperature. Films were exposed to membrane at –80 °C and developed.

Inhibition of C/EBPα degradation by proteasomal inhibitors

BALB/MK2 cells were transfected with pcDNA3-C/EBPα. Forty-six hours following transfection, cells were incubated with either 25 μM MG-132 or 10 μM lactacystin for 30 min prior to the addition of 50 μg/ml cycloheximide. Lysates were prepared at the indicated time points and subjected to western blot analysis. MG-132 was prepared as a 25 mM stock solution in Me_2SO, and lactacystin was prepared as a 5 mM stock solution in Me_2SO. Control experiments were carried out with Me_2SO.

In vitro proteasomal degradation assay

Degradation reactions were carried out in a final volume of 10 μl and contained 30 μg of S-100 fraction as the source of ubiquitin-proteasomal system components. The reaction mixtures contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP along with nucler extracts from pcDNA3-C/EBPα transfected BALB/MK2 cells as a proteasomal substrate (125 ng/reaction) supplemented with energy regeneration system (ERS) and 1 mM ubiquitin. For the inhibition of proteasomal activity, 25 μM of MG-132 or 10 mM LiCl was included. Reactions were incubated at 37 °C for 2 hr and were terminated by boiling after the addition of an equal volume of 2× SDS sample buffer. The reaction products were resolved by 10% SDS-PAGE and subjected to

western blot analysis

Effect of Li^+ on C/EBPα stability

BALB/MK2 cells were transfected with pcDNA3-C/EBPα and treated with 20 mM lithium chloride as described above. Twenty-two hours after the lithium treatment, cells were incubated with 50 μg/ml cycloheximide. Cells were harvested at various time points and extracts were subjected to western blot analysis followed by densitometric analysis.

Detection of ubiquitinated C/EBPα

For the detection of ubiquitinated C/EBPα in the cell, BALB/MK2 keratinocytes were plated on 60 mm culture dish and transfected with pCMV-Flag-C/EBPα. Twenty-four hours later transfected cells were treated with 20 mM LiCl for 24 hours. For MG-132 treatment, cells were incubated with 25 μM MG-132 for 2 hours. Forty-eight hours following transfection, cells were washed with ice-cold PBS, lysed in ice-cold immunoprecipitation buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM PMSF, 5 mM N-ethylmaleimide and Mini Complete protease inhibitor tablet from Roche Molecular Biochemicals) and rocked for 20 min at 4 °C. N-ethylmaleimide was included to inhibit isopeptidase activity. The lysed cells were scraped and centrifuged at 14000g for 10 min at 4 °C. Anti-Flag monoclonal antibody and protein A/G-agarose plus were added to cleared lysates and rocked overnight at 4 °C. Immunoprecipitates were centrifuged at 2500g and washed three times with ice-cold lysis buffer. Immunoprecipitated proteins were boiled in 2X SDS sample buffer, electrophoresed and analyzed by western blot. In vitro ubiquitination reactions were carried out in a final volume of 15 μl and contained 40 μg of S-100 fraction. The reaction mixtures

contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP along with nucler extracts from pCMV-Flag-C/EBPα transfected BALB/MK2 cells as a substrate (2 μg/reaction) supplemented with energy regeneration system (ERS), 1 mM ubiquitin and 10 μM ubiquitin-aldehyde. To inhibit proteasomal activity, 25 μM of MG-132 was included in reaction mixture. Reactions were incubated at 37 °C for 2 hr and were terminated by adding 5 μl of 4 % SDS. Reaction mixture was diluted 130 μl of immunoprecipitation buffer and Flag-C/EBPα was immunoprecipitated using anti-Flag antibody. Immunoprecipitated proteins were boiled in 2X SDS sample buffer, electrophoresed and analyzed by western blot.

Site-Directed Mutagenesis

Threonine to alanine mutations on T222 and T226 of pcDNA3-C/EBPα (T^{222} PPP T^{226}PVPS^{230}P) were introduced using Quick-change mutagenesis kit (Stratagene) according to manufacturer's protocol. The following primer was used to generate the threonine to alanine mutations in T222226A. [5'-G^{648}CAGCCTGGCCACCCT(A→G)CGCCGCCGCCG(A→G)CGCCCGTGCCCAGC CCTC^{694}-3']. Nucleotide changes from cDNA sequence are indicated. Specific mutations were confirmed by sequencing.

RESULTS

C/EBPα is a short-lived protein

Numerous proteins involved in regulation of the cell cycle are short-lived proteins that are degraded via an ubiquitin-dependent proteasomal pathway. Because C/EBPα is involved in the regulation of mitotic growth arrest and/or differentiation we examined the half-life of C/EBPα protein and whether proteasomal inhibitors can increase half-life of the C/EBPα protein. BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 46 hours later cells were treated with cycloheximide in the presence or absence of proteasomal inhibitor. Cells were collected at 0, 0.5, 1 and 2 hours post cycloheximide treatment and C/EBPα protein levels were determined by western blotting. As shown in Fig. 1A, treatment of BALB/MK2 keratinocytes with proteasome inhibitor, MG-132, blocked the degradation of C/EBPα protein. Treatment of BALB/MK2 cells with lactacystin, a highly specific proteasome inhibitor (36), also blocked degradation of C/EBPα protein (Fig. 1B). Densitometric analysis of these blots revealed C/EBPα has a half-life of approximately 1 hour (Fig. 1C). These results demonstrate that C/EBPα is short-lived protein and suggest that C/EBPα is degraded via a proteasomal mechanism.

C/EBPα is ubiqutinated and is a proteasome substrate

Since proteasome substrates are often polyubiquitinated before their degradation, we examined whether C/EBPα is ubiquitinated in BALB/MK2 cells. BALB/MK2 keratinocytes were transfected with Flag-tagged C/EBPα and 46 hours later cells were left untreated or treated with proteasome inhibitor, MG-132 for 2 hours. Flag immunoprecipitates were prepared from pCMV-Flag C/EBPα transfected BALB/MK2 cells by using monoclonal antibodies to Flag, followed by immunoblot with polyclonal

antibody to ubiquitin. As shown in Fig. 2A (left panel), ubiquitin-immunoreactive higher molecular weight forms were detected in Flag-C/EBPα transfected cell lysates and treatment of cells with MG-132 further increased ubiquitin-reactive higher molecular weight forms of C/EBPα. Membrane was stripped and reprobed with an antibody to C/EBPα. As shown in Fig. 2A (right panel), high molecular weight immunoreactive forms of C/EBPα were detected. To provide additional evidence for the proteasomal mediated degradation of C/EBPα, we examined whether that C/EBPα is degraded via the proteasome and ubiquitinated in a cell free assay. HeLa S-100 fraction contains proteasome and ubiquitin ligases and can be used to demonstrate whether a protein is degraded via an ubiquitin-proteasome pathways. As shown in Fig. 2B, C/EBPα was degraded by HeLa S-100 fraction in an ATP- and ubiquitin-dependent manner. When MG-132 was included in the assay, it blocked the degradation of the C/EBPα protein. While C/EBPα protein was not degraded in the absence of ATP and ubiquitin, its electrophoretic mobility was increased suggesting C/EBPα is a substrate for cellular phosphatases in vitro. In order to examine whether C/EBPα is ubiquitinated in vitro, Flag-tagged C/EBPα protein was incubated with HeLa S-100 fraction for 2 hours with/without MG-132 in the presence of ubiquitin-aldehyde, an isopeptidase inhibitor. Flag-tagged C/EBPα protein was immunoprecipitated from in vitro ubiquitination mixture and subjected to immunoblot with ubiquitin antibody. As shown in Fig. 2C (left panel), ubiquitin-immunoreactive higher molecular weight forms of C/EBPα were detected and MG-132 further increased ubiquitin-immunoreactive higher molecular weight forms of C/EBPα. Similar results were obtained when membrane was stripped and reprobed with an antibody to C/EBPα (Fig. 2C, right panel). Collectively, these results demonstrate that C/EBPα is degraded via an ubiquitin-

dependent proteasomal pathway.

Lithium increases C/EBPα protein levels

Ross et al (32) showed that GSK3 is a C/EBPα kinase and phosphorylates T222 and T226 of C/EBPα and lithium treatment can block this phosphorylation. Because phosphorylation by GSK3 is known to target cell cycle regulatory proteins such as p21 (26), β-catenin (27), cyclin D1 (28) and c-myc (29) for proteasomal degradation, we examined whether treatment with lithium, an inhibitor of GSK3 (30,31), can block C/EBPα degradation. BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and treated with lithium chloride, a known inhibitor of GSK3. Lithium treatment produced a dose dependent increase in C/EBPα protein levels (4-5 fold increase at 20 mM LiCl) as well an electrophoretically faster migrating form of C/EBPα protein (Fig. 3A and 3B). This increase in electrophoretic mobility is consistent with lithium inhibiting GSK3-mediated phosphorylation of C/EBPα. To confirm this notion, lysates from untreated cells were incubated with protein phosphatase prior to electrophoresis. As shown in Fig. 3C, phosphatase treatment, like lithium, resulted in a faster migrating form of C/EBPα. However, phosphatase treatment produced a faster migrating form of C/EBPα than lithium treatment suggesting that there are additional phosphorylation sites within C/EBPα that are not sensitive to lithium treatment. The effect of lithium on C/EBPα protein levels was specific as treatment with sodium, another cationic metal, did not increase C/EBPα levels (Fig. 3D). In contrast to the effect of lithium on C/EBPα protein levels, lithium had no effect on C/EBPα mRNA levels indicating that the effects of lithium are post transcriptional (Fig. 3E). Lithium treatment of untransfected BALB/MK2 cells also resulted in an increase in the endogenous C/EBPα levels, produced a faster migrating form of C/EBPα and had no

effect on C/EBPα mRNA levels (data not shown). The fact that lithium treatment produced an increase in C/EBPα protein but not C/EBPα mRNA in both C/EBPα transfected and untransfected cells indicates that the effects of lithium are promoter independent (chromatin embedded C/EBPα promoter versus the CMV promoter of pcDNA3- C/EBPα) and that the effects of lithium are post-transcriptional.

Lithium-induced increase in C/EBPα protein levels is accompanied by an increase in C/EBPα transactivation activity

To ascertain if the lithium-induced increase in C/EBPα protein was also accompanied by an increase in C/EBPα transactivation activity we transfected BALB/MK2 keratinocytes with C/EBPα and a luciferase reporter gene fused to the C/EBP-dependent myelomonocytic growth factor promoter (MGF82) and then treated these cells with lithium. As shown in Fig. 4A, lithium treatment, but not sodium treatment resulted in a 10-fold increase in pMGF-82 reporter activity over that observed in cells not treated with lithium. This increase in reporter activity was accompanied by an increase in C/EBPα protein levels (Fig. 4B). In contrast, lithium treatment did not produce an increase in MGF-40 reporter activity, reporter plasmid which lacks C/EBP sites, demonstrating that C/EBP binding sites are required for the lithium response (data not shown). pMGF-82 reporter activity was also increased in cells treated with lithium but not transfected with C/EBPα suggesting that lithium induced increases in endogenous C/EBPα are also accompanied by increases in C/EBPα transactivation activity. Since lithium is also known to inhibit inositol monophosphatase, we treated keratinocytes with 10 mM myoinositol and lithium together or L-690,330 alone. L-690,330 is a potent inhibitor of inositol monophosphatase. Myoinositol and L-690,330 had no effect on the transactivation activity of C/EBPα and indicating that the

observed effect of lithium is not due to inhibition of inositol monophosphatase (data not shown). The above results suggest that while lithium inhibits GSK3-mediated phosphorylation of C/EBPα, this is not associated with changes in transactivation activity of C/EBPα protein.

Lithium increases C/EBPα protein levels in GSK3 independent manner.

To test the idea lithium produces an increase in C/EBPα protein by inhibiting GSK3 mediated phosphorylation of C/EBPα and subsequent proteasomal degradation, we mutated the GSK3 sites in C/EBPα to produce a mutant protein with T222A and T226A amino acid substitutions. If GSK3 mediated phosphorylation of C/EBPα targets it for proteasomal degradation then in untreated cells the T222A/T226A C/EBPα mutant protein should display increased levels compared to wild type protein. Moreover, in lithium treated cells, the mutant protein should not be increased. BALB/MK2 keratinocytes were transfected with mutant or wild type C/EBPα and then treated with lithium. Lithium treatment produced a similar in increase in both the wild type and mutant protein (Fig. 5A). Moreover, T222A/T226A C/EBPα protein levels were similar to wild-type protein levels in the untreated cells. Wild type C/EBPα protein demonstrated an electrophoretic mobility shift as described above, however the mutant protein did not exhibit a electrophoretic mobility shift suggesting it was no longer a GSK3 substrate (Fig. 5B). These results suggest that while lithium inhibits GSK3-mediated phosphorylation on C/EBPα, the effect of lithium on C/EBPα protein is through a GSK3 independent mechanism. To confirm this notion, we treated cells with GSK inhibitors SB216763 and SB415286 which have been reported to be specific inhibitors of GSK3 (37). Unlike lithium neither SB216763 nor SB 415286 increased C/EBPα protein levels (Fig. 5C).

Lithium increases the half-life of the C/EBPα protein by direct inhibition of proteasome activity

As the effect of lithium on C/EBPα protein levels were post-transcriptional and GSK3 independent, we examined whether lithium directly affects stability of C/EBPα protein. To determine whether lithium can alter the stability of C/EBPα protein we treated C/EBPα transfected cells with cycloheximide in the presence or absence of lithium. As shown in Fig. 6A and 6B, lithium treatment inhibited C/EBPα protein degradation and greatly increased the stability and the half-life of C/EBPα. In order to determine whether lithium induces ubiquitination of C/EBPα, we transfected BALB/MK2 keratinocytes with Flag-tagged C/EBPα and 24 hours later cells were treated with 20 mM LiCl for 24 hours. Flag immunoprecipitates were prepared from pCMV-Flag C/EBPα transfected BALB/MK2 cells by using monoclonal antibodies to Flag, followed by immunoblot with polyclonal antibody to ubiquitin. As shown in Fig. 6C, lithium treatment induced ubiquitin-immunoreactive higher molecular weight forms of C/EBPα. We next examined the effect of lithium on proteasome-mediated degradation of C/EBPα in vitro using HeLa S-100 fraction. As shown in Fig. 6D, the addition of lithium or the proteasome inhibitor, MG-132 to HeLa S-100 fraction directly inhibited the degradation of C/EBPα in vitro. Collectively these results suggest that lithium increases C/EBPα protein levels through direct inhibition of proteasome activity. In support of this finding are recent studies by Rice and Sartorelli (34) demonstrating that LiCl specifically inhibits the chymotryptic-like activity of the 20S and 26 S proteasome. To extend the role of lithium as a general proteasomal inhibitor, we examined the effect of lithium on p53, as p53 is degraded via the ubiquitin-proteasome pathway (38). Similar to C/EBPα results, lithium treatment increased p53 levels in

BALB/MK2 cells and blocked the in vitro degradation of p53 (Fig. 7A and 7B).

DISCUSSION

In the present study we observed that C/EBPα is short-lived protein with half-life of approximately 1 hour and that treatment with proteasome inhibitors, MG-132 or lactacystin, block C/EBPα protein degradation. We provide evidence that C/EBPα is poly-ubiquitinated in BALB/MK2 cells and the results of our *in vitro* studies demonstrate that C/EBPα is degraded via the proteasome in an ATP- and ubiquitin-dependent manner. This is first demonstration that the C/EBPα transcription factor is degraded via ubiquitin-proteasomal pathway in mammalian cells. C/EBPα is an important regulator of cell growth and/or differentiation in numerous cell types. In addition to its function as a transcription factor, C/EBPα has been reported to negatively regulate cell proliferation through a transcription-independent mechanism in which C/EBPα forms a complex with cdk2 and cdk4 preventing cyclin/cdk complex formation and cell cycle progression (18). Regulation of C/EBPα protein levels through an ubiquitin-proteasomal pathway would serve to control both the transcription-dependent and transcription-independent activities of C/EBPα. Recently C/EBPα, C/EBPβ, C/EBPδ and C/EBPε have been shown to undergo sumoylation and covalent modification of C/EBPε by SUMO1 appears to activate C/EBPε transactivaiton function but does not target C/EBP for degradation (39,40). Thus, C/EBPα transcription activity and protein level can be modulated by covalent modification involving sumoylation and ubquitination. Whether proteasomal degradation of C/EBPα can be regulated in a cell cycle dependent manner or through C/EBPα phosphorylation via sites other than GSK3 sites is not known. However, in some tissues/cells C/EBPα protein levels are often disparate with mRNA levels suggesting C/EBPα protein levels are regulated at the translation and/or post-translational level (2,6).

Lithium has been successfully used as a therapeutic agent for bipolar disorder and has multiple effects on embryonic development and pattering, glycogen synthesis, hematopoiesis and other cellular processes (31). We observed that lithium treatment of BALB/MK2 keratinocytes resulted in a five-fold increase in C/EBPα protein. Generally, lithium is considered to produce its biological effects through the inhibition of GSK3 or inositol monophosphatase. Because phosphorylation by GSK3 is known to target cell cycle regulatory proteins such as p21 (26), β-catenin (27), cyclin D1 (28) and c-myc(29) for proteasomal degradation, we examined whether effect of lithium on C/EBPα levels was mediated through inhibition of GSK3. While GSK3 is a C/EBPα kinase and has been shown to phosphorylate T222 and T226 of C/EBPα (32), our results with mutant C/EBPα (T222A/T226A) as well as with pharmacological inhibitors of GSK3, SB216763 and SB415286, indicate that the effect of lithium on C/EBPα protein levels is independent of GSK3. Moreover inhibitors of inositol monophosphatase, unlike lithium treatment had no effect on C/EBPα transactivation activity suggesting that the effects of lithium involve cellular targets other than inositol monophasphatase or GSK3.

Recently, Rice and Sartorelli demonstrated that lithium chloride is a specific inhibitor of the chymotrptic activity of both the 20 S and 26 S proteasome (34). In BALB/MK keratincytes, we observed that lithium treatment increases the half-life of C/EBPα 6-fold and that lithium directly inhibits proteasome activity and blocks C/EBPα degradation in vitro. Taken together these results indicate that lithium is increasing C/EBPα protein levels in BALB/MK2 cells by directly inhibiting proteasomal activity. Overexpression of C/EBPα in BALB/MK2 keratinoytes inhibits cell proliferation (13) and we have observed that lithium treatment of BALB/MK2 cells

significantly inhibits thymidine incorporation into DNA (unpublished MS and RCS). Thus, it is possible that the lithium induced increase in C/EBPα is associated with inhibition in cell proliferation, however, additional studies are required to understand this effect as other cell cycle regulatory proteins such as p21, β-catenin, cyclin D1 and c-myc are altered by lithium treatment. Recently Mao et al have shown that lithium treatment of bovine endothelial cells causes the accumulation of p53 protein without affecting p53 mRNA levels (41). We have confirmed their findings in BALB/MK2 keratinocytes and have extended these results to by showing that lithium directly inhibits the proteasomal degradation of p53.

Lithium-induced increase in C/EBPα protein was accompanied by comparable increases in C/EBPα transactivation activity. We also observed that the T222A/T226A mutant C/EBPα protein displayed similar increases in transactivation activity and protein level as wild type C/EBPα in the presence of lithium, indicating that inhibition of GSK3 mediated phosphorylation of C/EBPα does not significantly alter C/EBPα transactivation activity. In contrast to C/EBPα, lithium treatment had little to no effect on C/EBPβ protein level, however lithium treatment did increase C/EBPβ transactivation activity in C/EBPβ transfected keratinocytes (unpublished MS, RCS). This observation is consistent with a recent report where growth hormone-induced dephosphorylation of C/EBPβ, presumbly mediated by a PI3K/AKT/GSK3 pathway resulted in increased C/EBPβ DNA binding activity (42).

GSK3 has an important role in the Wnt signaling pathway where it along with other proteins regulates the phosphorylation and degradation of β-catenin. Recently, Ross et al have shown that Wnt signaling results in the inhibition of adipogeneisis and this inhibition of adipogenesis is associated with the loss of expression of C/EBPα and

PPARγ mRNA and protein, two transcription factors that are important in adipogenesis (33). Importantly, lithium treatment also inhibits adipogenesis (32). Thus, in 3T3-L1 cells, Wnt signaling or lithium treatment (Wnt mimic) results in decreased expression of C/EBPα while in keratinocytes we observed that lithium treatment produces an increase in C/EBPα protein levels. This apparent difference could be due to a differential response between the two cell types or it is possible that Wnt and lithium treatment block adipogenesis upstream of the expression of C/EBPα and PPARγ thus masking an effect of lithium on C/EBPα protein stability.

In conclusion our results demonstrate that C/EBPα is degraded via ubiquitin-dependent proteasomal pathway and that lithium stabilizes C/EBPα through direct inhibition of proteasome activity in a GSK3-independent manner. In addition to the effect of lithium on inositol monophosphatases and GSK3, the inhibition of proteasome activity by lithium is another mechanism that should be considered when lithium is employed in biological systems.

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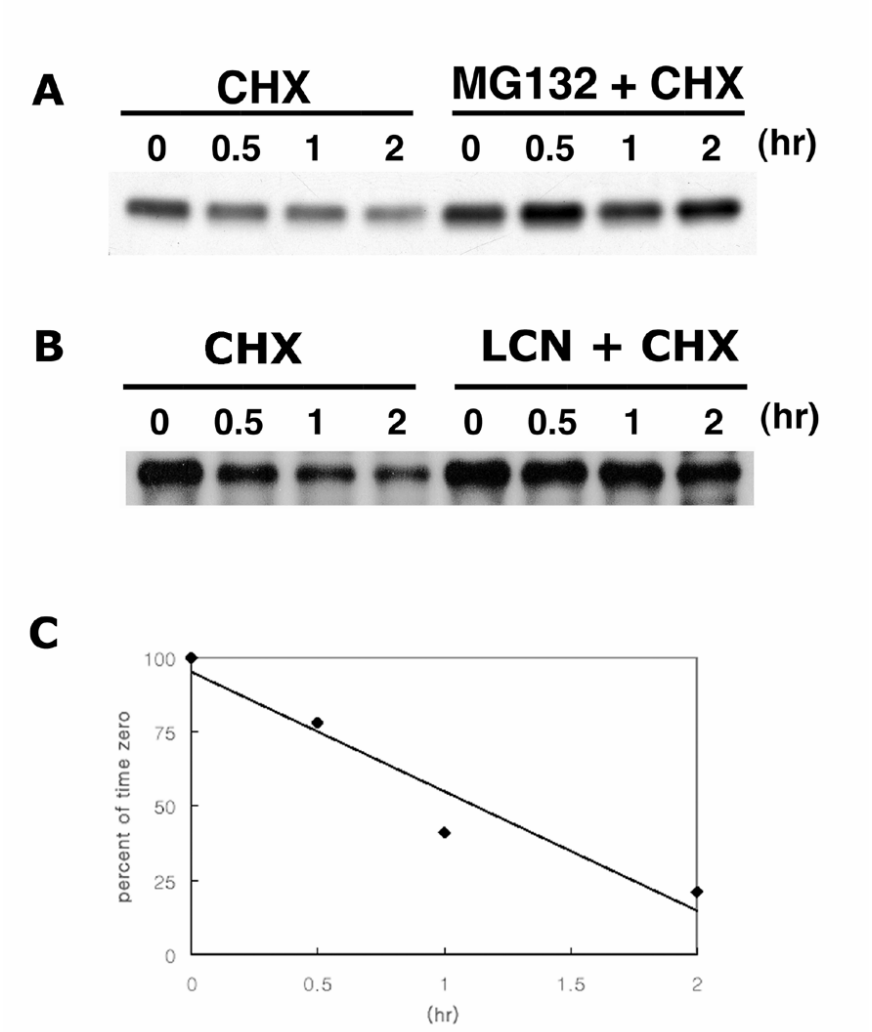
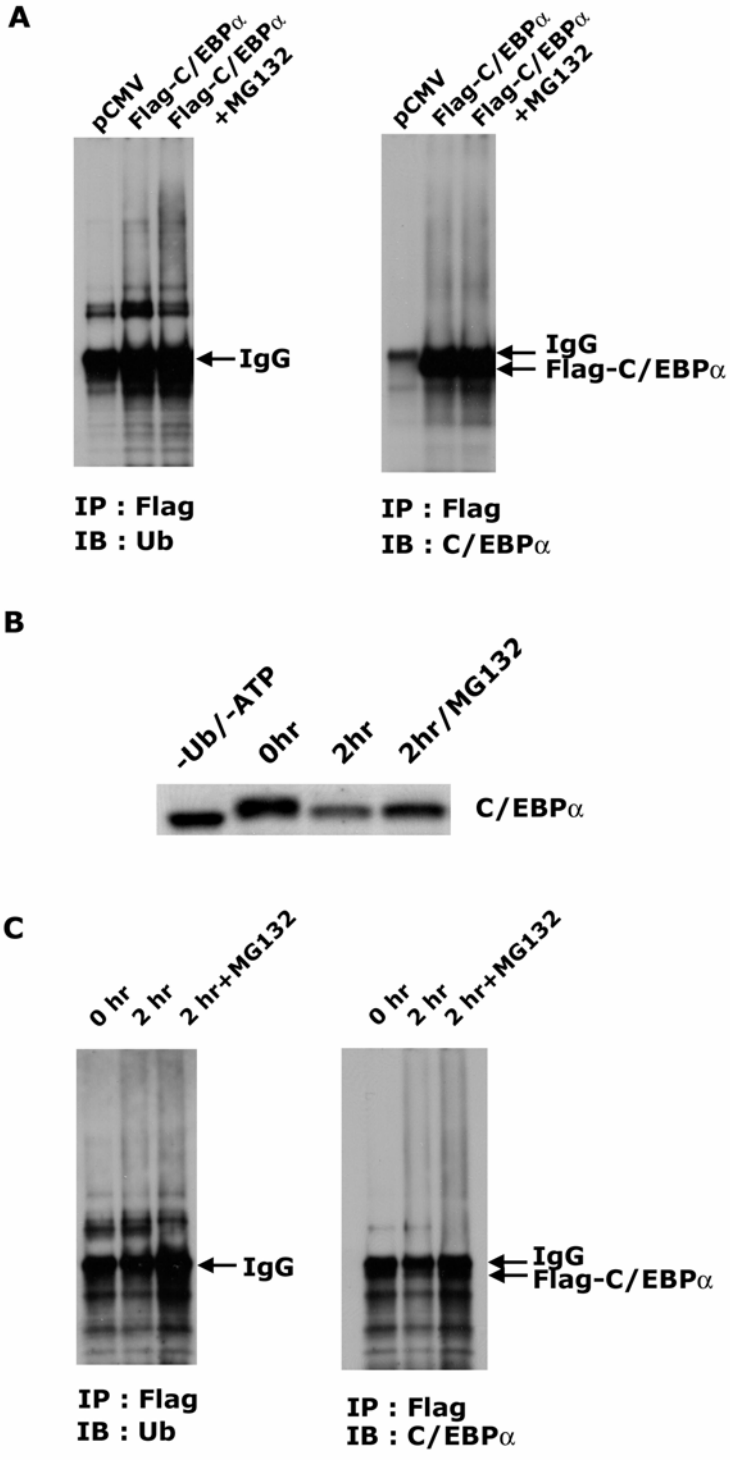


Fig. 1 *C/EBPα is degraded via protesome.*

A/B) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 46 hours later were treated cycloheximide with/without MG-132 or lactacystin (LCN). Whole cell lysates were prepared at the indicated times and equal amounts of protein were subjected to western blot analysis. C) Densitometric analysis of results from Figure 1A and B expressed as % of time zero. (N=3/time point, average coefficient of variation <15%, r^2=0.93)


A
pCMV
Flag-C/EBPα
Flag-C/EBPα
+MG132
IgG
IP : Flag
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pCMV
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+MG132
IgG
Flag-C/EBPα
IP : Flag
IB : C/EBPα
B
-Ub/-ATP
0hr
2hr
2hr/MG132
C/EBPα
C
0 hr
2 hr
2 hr+MG132
IgG
IP : Flag
IB : Ub
0 hr
2 hr
2 hr+MG132
IgG
Flag-C/EBPα
IP : Flag
IB : C/EBPα

Fig. 2 *C/EBPα is ubiquitinated and is a proteasome substrate.*

A) BALB/MK2 cells were transfected with Flag-tagged C/EBPα and 46 hours later cells were treated with 25 μM MG-132 for 2 hours. Lysates were immunoprecipitated with Flag monoclonal antibody, followed by western blot with ubiquitin antibody (left panel). Membrane was stripped and reprobed with C/EBPα antibody(right panel). Arrows indicate IgG heavy chain and Flag-tagged C/EBPα. B) C/EBPα was added to reaction mixtures and degradation was assayed by western blotting with C/EBPα antibody. The reaction mixtures contained HeLa S-100 fraction, ubiquitin, ERS (energy regeneration system), and ATP and were incubated for 2 hours at 37 ^{o}C except for the 0 hr reaction, in which reaction was terminated at t=0. For inhibition of proteasomal activity, the reaction mixture was incubated with 25 μM of MG-132. C) Flag-tagged C/EBPα protein was immunoprecipitated with Flag monoclonal antibody from in vitro ubiquitination reaction, followed by western blot with ubiquitin antibody. Membrane was stripped and reprobed with C/EBPα antibody. Arrows indicate IgG heavy chain and Flag-tagged C/EBPα.

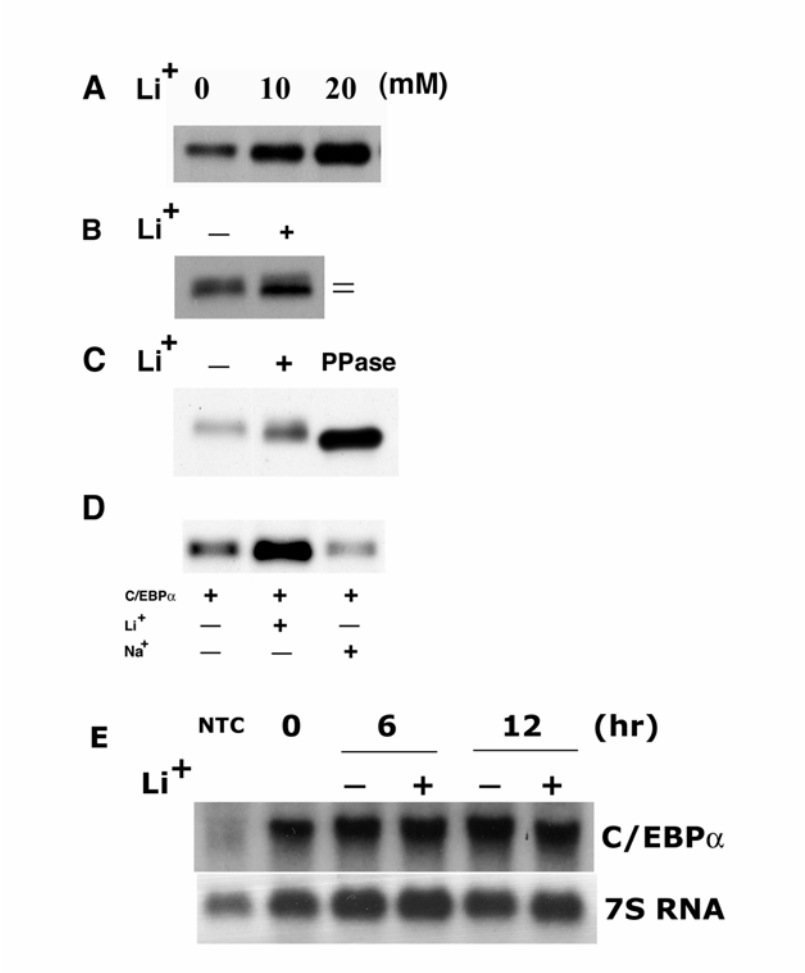

Fig. 3 *Effect of lithium on C/EBPα protein*.

A) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 24 hours later were treated with 20 mM LiCl. Twenty-four hours later cell lysates were prepared and equal amounts of protein were subjected to western blot analysis. B) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 24 hours later were treated with 20 mM LiCl. Twenty-four hours later cell lysates were prepared and proteins were separated for western blot analysis. Different amount of protein was loaded to attempt to equalize the amount of C/EBPα protein. lane 1 : 3 μg of nuclear

extracts from untreated cells, lane 2: 1 μg of nuclear extracts from 20 mM LiCl-treated cells. C) Lysates from untreated pcDNA3-C/EBPα transfected cells were incubated with λ phosphatase prior to SDS-PAGE. lane 1 : 3 μg of nuclear extracts from untreated cells, lane 2: 1 μg of nuclear extracts from 20 mM LiCl-treated cells, lane 3 : 10 μg of λ-phosphatase treated nuclear extracts. D) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 24 hours later were treated with 20 mM LiCl or 20 mM NaCl. Twenty-four hours later cell lysates were prepared and equal amounts of protein were subjected to western blot analysis. E) Twenty-four hours following transfection with pcDNA3-C/EBPα, BALB/MK2 cells were treated with 20 mM LiCl and RNA was isolated at the indicated times. 15 μg of total RNA was loaded per each well and northern blot analysis was conducted. Membrane was stripped and reprobed for 7S RNA to confirm equal loading.

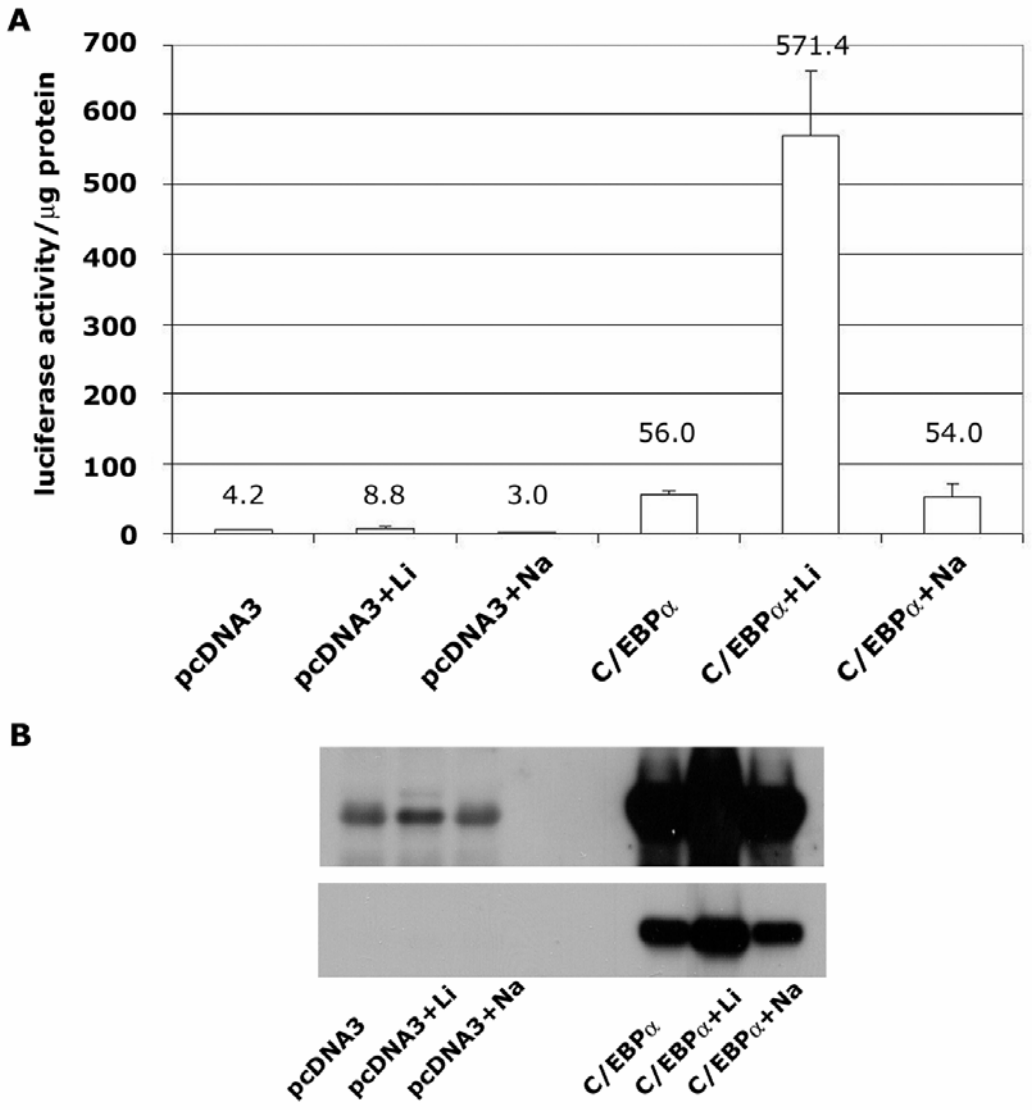

Fig. 4 Lithium-*induced increase in C/EBPα levels is accompanied by an increase in C/EBPα transactivation activity.*

A) BALB/MK2 keratinocytes were transfected with pcDNA3 and pMGF82 reporter plasmids or with pcDNA3-C/EBPα and pMGF82 reporter plasmids and 24 hours later were treated with 20 mM LiCl or 20 mM NaCl. Twenty-four hours later cells were

lysed with cell culture lysis buffer and luciferase activity was determined. Luciferase activity was normalized to protein levels and each value represents the mean ± SD of triplicates per treatment. B) C/EBPα protein levels from the same experiment shown in Fig. 4A. Same blot was differentially exposed to visualize endogenous C/EBPα and C/EBPα in pcDNA3-C/EBPα transfected cells.

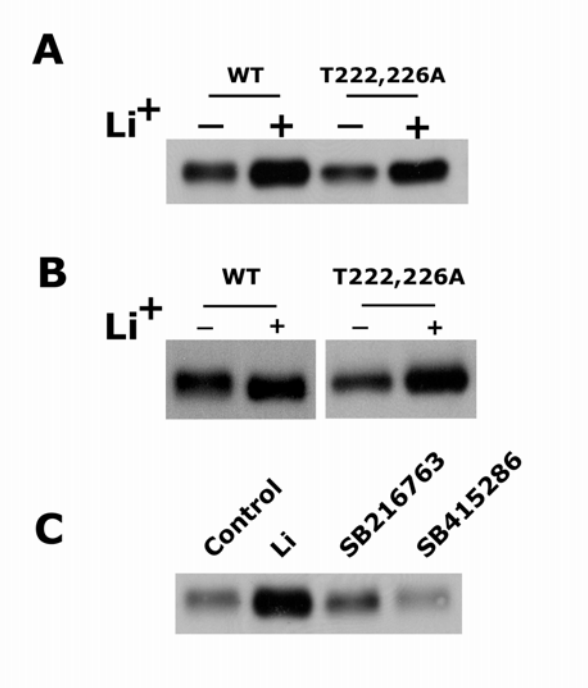

Fig. 5 *Effect of lithium on C/EBPα protein level is GSK3 independent.*

A) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα or mutant pcDNA3-C/EBPα T222,T226A and 24 hours later were treated with 20 mM LiCl. Twenty-four hours later cell lysates were prepared and equal amounts of proteins were subjected to western blot analysis. B) Lysates were prepared as described above in 5A except proteins were separated on an 8% tris-glycine gel. Different amount of protein was loaded to attempt to equalize the amount of C/EBPα protein. lane 1, 3 : 3 μg of nuclear extracts from untreated cells, lane 2, 4: 1 μg of nuclear extracts from 20 mM LiCl-treated cells. C) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 24 hours later were treated with 20 mM LiCl, 20 μM SB216763, or 40 μM SB415286. Twenty-four hours later cell lysates were prepared and equal amounts of protein were subjected to western blot analysis.

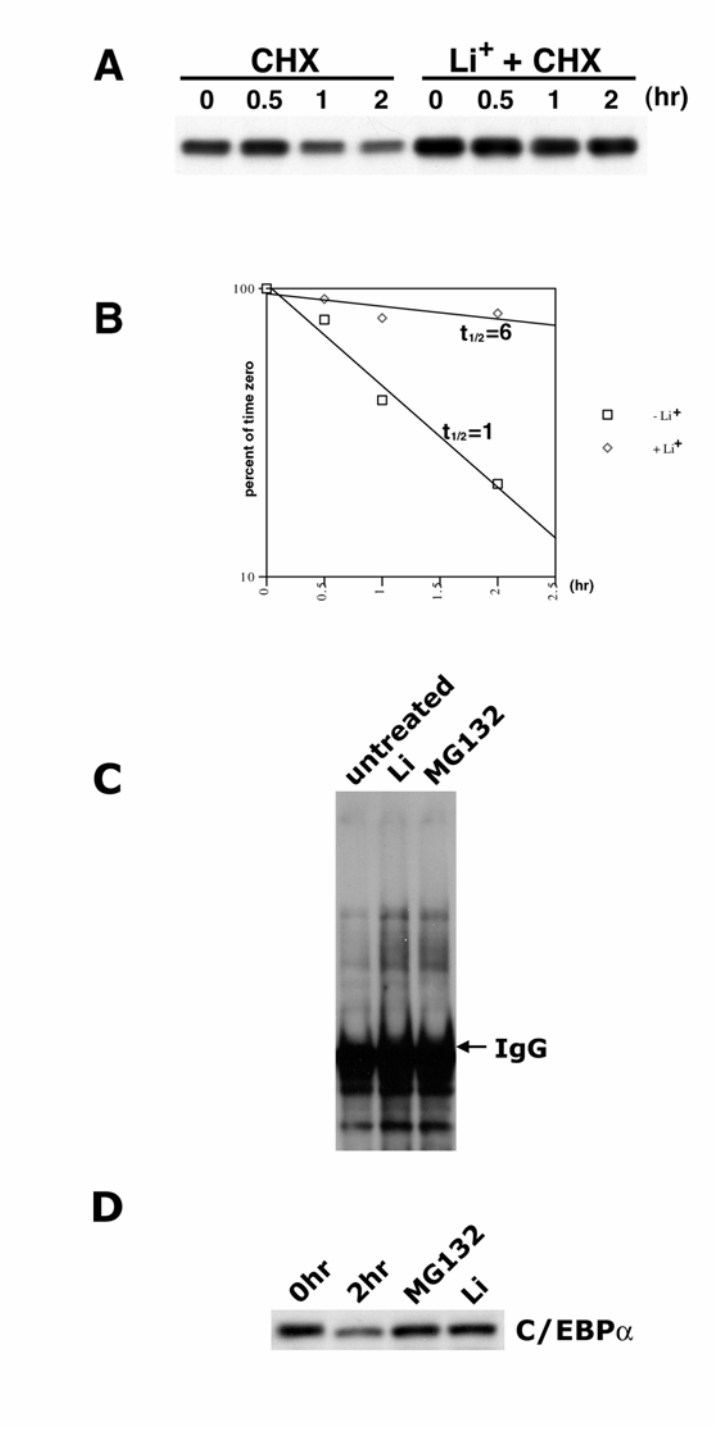
A
CHX
Li+ + CHX
0 0.5 1 2 0 0.5 1 2 (hr)
B
100
10
percent of time zero
t1/2=6
t1/2=1
- Li+
+ Li+
0 0.5 1 1.5 2 2.5 (hr)
C
untreated
Li
MG132
IgG
D
0hr
2hr
MG132
Li
C/EBPα

Fig. 6 *Lithium stabilizes C/EBPα protein by interfering with proteasomal activity.*

A) BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPα and 24 hours later were treated with or without 20 mM LiCl. Forty-six hours post transfection, cells were treated with 50 μg/ml cycloheximide and whole cell lysates were prepared at the indicated times and equal amounts of protein were subjected to western blot analysis. B) Densitometric analysis of results from Figure 6A expressed as % of time zero. (N=3/time point, average coefficient of variation <15%, r^2=0.93, r^2=0.65) C) BALB/MK2 cells were transfected with Flag-tagged C/EBPα. Twenty-four hours later transfected cells were treated with 20 mM LiCl for 24 hours. For MG-132 treatment, cells were incubated with 25 μM MG-132 for 2 hours. Lysates were immunoprecipitated with Flag monoclonal antibody, followed by western blot with ubiquitin antibody. D) C/EBPα was added to reaction mixtures and degradation was assayed by western blotting with C/EBPα antibody. The reaction mixtures contained HeLa S-100 fraction, ubiquitin, ERS (energy regeneration system), and ATP and were incubated for 2 hours at 37 ^{o}C except for the 0 hr reaction, in which reaction was terminated at t=0. For inhibition of C/EBPα degradation, the reaction mixture was incubated with 25 μM of MG-132 or 10 mM LiCl.

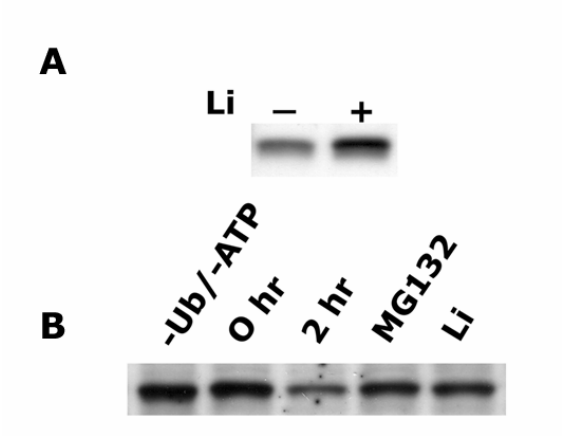

Fig. 7 *Lithium treatment increases p53 levels in BALB/MK2 cells and blocks p53 degradation by HeLa S-100 fraction.*

A) BALB/MK2 keratinocytes were transfected with p53 expression vector and 24 hours later were treated with 20 mM LiCl. Twenty-four hours later cell lysates were prepared and equal amounts of protein were subjected to western blot analysis. B) In vitro p53 proteasomal degradation assay. p53 was added to reaction mixtures and degradation was assayed by western blotting with p53 antibody. The reaction mixtures contained HeLa S-100 fraction, ubiquitin, ERS (energy regeneration system), and ATP and were incubated for 2 hours at 37 ^{o}C except for the 0 hr reaction, in which reaction was terminated at t=0. For inhibition of p53 degradation, the reaction mixture was incubated with 25 μM of MG-132 or 10 mM LiCl.

MANUSCRIPT II

C/EBPα Expression is Diminished in Skin Squamous Cell Carcinomas (SCCs) and Re-expression in SCC Cell Lines Inhibits Proliferation.

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Abbreviations; C/EBPα: CCAAT/enhancer binding protein-alpha

ABSTRACT

CCAAT/enhancer binding protein alpha (C/EBPα) is involved in mitotic growth arrest and differentiation in numerous cell types and C/EBPα has been implicated as a human tumor suppressor in acute myeloid leukemia. We have previously shown that C/EBPα is expressed abundantly in mouse epidermal keratinocytes. In the current study, the expression of C/EBPα was evaluated in mouse skin squamous cell carcinoma lines. C/EBPα mRNA and protein levels were greatly diminished or undetectable in all seven SCC cell lines compared to normal keratinocytes. In contrast to C/EBPα, C/EBPβ protein was expressed at relatively normal levels in all SCC cell lines. Forced expression of C/EBPα in SCC cell lines resulted in the inhibition in SCC cell proliferation. Expression of C/EBPα also resulted in the expression of loricrin, a late stage marker of squamous differentiation. Treatment of SCC cell lines with 5'-aza-deoxycytidine, an inhibitor of methylation of cytosine residues in DNA, increased C/EBPα expression in some cell lines suggesting the C/EBPα promoter region may be transcriptionally silenced by hypermethylation. To determine whether the decrease in C/EBPα expression observed in the SCC cell lines occurs in the primary cancer lesion itself, the immunohistochemical staining for C/EBPα in mouse skin squamous cell carcinomas was conducted. C/EBPα protein expression was negligible in all 14/14 SCC examined compared to normal epidermis. These results suggest the loss of C/EBPα expression may contribute to the altered growth and differentiation characteristics of skin SCCs.

INTRODUCTION

The CCAAT/enhancer binding proteins (C/EBP) are members of the basic leucine zipper (bZIP) class of transcription factors that contain a basic DNA binding domain and a leucine zipper domain involved in homo- or heterodimerization.(1, 2) There are six members of the C/EBP family [C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ] (3, 4). C/EBPα plays an important role in control of cell proliferation as well as differentiation in a variety of cell types (5). C/EBPα has been proposed to regulate p21 expression as well as to directly interact and enhance the ability of p21 to inhibit cdk2 (6, 7). C/EBPα has also been proposed to inhibit cell growth through its interaction with Rb family proteins (8, 9). C/EBPα can also directly repress E2F function through its physical association with E2F and this repression is necessary for growth arrest and adipocyte and granulocyte differentiation (10). Recent studies indicate that C/EBPα can block growth independent of its DNA binding and transcriptional activity by forming a complex with cdk2 and cdk4 and thereby blocking cyclin-cdk interactions and cell cycle progression (11). C/EBPα is inactivated by mutation or through its association with oncoprotein AML-1-ETO in human acute myeloid leukemia (12, 13). The inactivation of C/EBPα is thought to result in differentiation block of the granulocytic blasts and has implicated C/EBPα as a tumor suppressor gene. C/EBPα expression is reduced in hepatocellular carcinomas and lung cancer cell lines suggesting a possible tumor suppressor function (14, 15).

We have previously shown that C/EBPα and C/EBPβ are abundantly expressed in mouse epidermal keratinocytes (16). In mouse keratinocytes, C/EBPβ is involved in the regulation of the early stages of squamous differentiation (17). Recently, C/EBPβ

has been shown to play a critical role in Ras-mediated mouse skin tumorigenesis and keratinocyte survival (18). Unlike C/EBPβ, C/EBPα does not cooperate with Ras to induce transformation of NIH 3T3 cells nor does it induce the expression of genes associated with the early stages of squamous differentiation in keratinocytes. However, the forced expression of C/EBPα in keratinocytes inhibits their growth (17) and preliminary data in mouse squamous cell carcinomas (SCCs) indicated that C/EBPα and C/EBPβ levels are reduced. Because C/EBPα is highly expressed in normal epidermis while its expression appears to be diminished in skin SCCs, we hypothesized that C/EBPα has a suppressor function in SCCs. We have examined C/EBPα levels in mouse skin SCC cell lines, SCCs, and keratoacanthomas and found that C/EBPα levels are significantly reduced in all lines and tumors. We also found that forced expression of C/EBPα induced growth arrest in SCC cell lines and expression of late differentiation marker, loricrin, in BALB/MK2 keratinocytes. Our results suggest that loss of C/EBPα is important in development of SCC.

MATERIALS AND METHODS

Cell lines and cell culture

Primary keratinocytes were isolated from newborn CD-1 mice (less than 3 days old) by overnight trypsin flotation at 4 °C. Isolated keratinocytes were plated at 6×10^6 cells/60 mm culture dish in Ca^{2+}-free EMEM supplemented with 10% non-Chelex-treated fetal bovine serum and 4 ng of hEGF per ml for 4 h to enhance cell attachment. Cultures were then gently washed with Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS) to remove any remaining calcium and unattached cells and then refed with low-calcium medium (Ca^{2+}-free EMEM supplemented with 4% Chelex-treated fetal bovine serum, 10 ng of hEGF per ml, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 250 ng of amphotericin B [Fungizone]/ml, with added calcium chloride to a final concentration of 0.05 mM). Medium was changed daily. Mouse SCC cell lines were isolated from various SCC that were induced by DMBA-initiation followed by TPA/or mirex promotion. MT2.5, MT2.6, MT2 3r3, T6, and M9.6 SCC cell lines were cultured in low-calcium medium without EGF (Ca^{2+}-free EMEM supplemented with 8% Chelex-treated fetal bovine serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, 250 ng of amphotericin B [Fungizone]/ml, and calcium chloride to a final concentration of 0.05 mM). FVBN-217 and TGAC-43 mouse skin SCC cell lines were kind gift from Dr. Ron Cannon (NIEHS, Research Triangle) and were cultured in high-calcium medium (DMEM supplemented with 10 % fetal bovine serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 250 ng of amphotericin B [Fungizone]/ml). BALB/MK2 keratinocytes (a gift from Dr. Weissman, University of North Carolina, Chapel Hill) were cultured in low-calcium medium with EGF (Ca^{2+} free EMEM supplemented with 8 % Chelex-treated fetal bovine serum, 4 ng of hEGF

per ml, and 0.05 mM calcium chloride).

Immunohistochemistry for C/EBPα and C/EBPβ

Paraffin-embedded tissue sections of normal skin and skin tumors derived from DMBA-initiation/TPA promotion were subjected to a antigen retrieval protocol (95 ^{o}C for 30 min in 10 mM citrate buffer pH 6.0) followed by incubation with anti-C/EBPα antibody (sc-61) or anti-C/EBPβ antibody (sc-150) (1:250, SantaCruz Biotechnology). A biotinylated goat anti-rabbit IgG was used as the secondary antibody. Detection was made by ABC kit (Vector Laboratories) and 3,3'-diaminobenzidine (DAB) as the chromagen (BioGenex). No C/EBPα or C/EBPβ staining was observed when the primary antibody was omitted and the control rabbit serum was applied.

Preparation of cell lysates

Nuclear extracts were prepared as previously described by Schreiber et al **(19)**. For the preparation of whole cell lysates, cells were washed with cold PBS, harvested by scraping, collected by brief centrifugation. Cells were lysed in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1mM PMSF, 100 μg/ml aprotinin, 100 μg/ml leupeptin, 1mM sodium orthovanadate, 0.6 % NP-40) by sonication and then one-tenth volume of 5 M NaCl was added. Incubation mixture was vortexed, incubated for 15 min on ice, and centrifuged (14,000g, 10 min, 4 ^{o}C). Supernatants were stored at –80 ^{o}C until use. Protein concentration was determined by the Bio-Rad protein assay reagent.

Western blot analysis

Equal amounts of whole cell lysate protein were precipitated by adding equal volume of 20% trichloroacetic acid and washed with acetone (-20 ^{o}C). Protein samples were solubilized and boiled in SDS sample buffer for 2 min, and then separated by SDS-

PAGE. The separated proteins were transferred to an Immobilon-P membrane (Millipore). Following incubation in blocking buffer (PBS with 1 % bovine serum albumin, 5 % non fat dry milk, and 0.1 % Tween-20) for 1 hr at room temperature, the membranes were probed for 2 hr at room temperature with rabbit polyclonal IgG raised against C/EBPα (sc-61, SantaCruz Biotechnology) or C/EBPβ (sc-150, SantaCruz Biotechnology). The membranes were washed and then probed with an HRP-linked secondary antibody for 1 hr at room temperature. Detection was made with an enhanced chemiluminescence reagent followed by exposure of membrane to film.

Northern blot analysis

Total RNA was isolated using Promega's SV total RNA isolation kit. C/EBPα cDNA was labeled with [α-32P] dCTP by using Ready-To-Go labeling beads (Amersham). RNA was electrophoresed on agarose gel containing formaldehyde and transferred to zeta-probe GT membrane (BioRad) and UV cross-linked. Blots were incubated at 65 °C in hybridization buffer (0.25 M Na_2HPO_4 pH 7.2, 7 % SDS) and sequentially washed with washing buffer 1 (20 mM Na_2HPO_4 pH 7.2, 5 % SDS) and washing buffer 2 (20 mM Na_2HPO_4, 1% SDS) at room temperature. Films were exposed to membranes at –80 °C and developed.

Transfection of SCC cell lines

SCC cell lines (MT2.5 and MT2 3r3) were transfected when they reached 30 to 40% confluence in 60-mm-diameter dishes with 2 μg of DNA (pcDNA3 or pcDNA3-C/EBPα) and 16 μg of Lipofectamine (Invitrogen). Transfection was performed in serum-free EMEM (containing 0.05 mM calcium) at 37°C and 5% CO_2 for 4 h, after which the cells were refed with low-calcium medium without EGF (Ca^{2+}-free EMEM supplemented with 8 % Chelex-treated fetal bovine serum and calcium chloride to a

final concentration of 0.05 mM). Forty-eight hours later, the cultures were split (1:3) and replated in the above medium containing 300 μg/ml G418 and this selection medium was changed every other day. On days 3, 5, and 7 after G418 selection, the total number of colonies in 30 random grid squares was counted and then converted to colonies per dish. The number of cells per colony was scored directly from 30 randomly chosen colonies.

5-aza-2'-deoxycytidine/ trichostatin A treatment

SCC cell lines were treated with different concentrations of 5-aza-2'-deoxycytidine (Sigma) as specified in the text for 72 hours. For co-treatment with trichostatin A, SCC cell lines were first treated with 1 μM 5-aza-2'-deoxycytidine for 48 hours and then treated with 1 μM 5-aza-2'-deoxycytidine and 0.3 μM trichostatin A (Sigma) for another 24 hours. Whole cell lysates were subjected to western blot analysis.

Double immunofluorescence

For immunofluorescence detection of C/EBPα and differentiation markers, BALB/MK2 cells were plated onto coverslips and transfected with pCMV-HA-C/EBPα plasimd. Forty-eight hours after transfection, the cultures were fixed in 4 % paraformaldehyde at 4 °C for 10 min and incubated with rabbit loricrin polyclonal antibody (Covance, 1:1,000) and HA monoclonal antibody (Roche, 1:2,000) in 0.2 % triton X-100 and 0.5% normal goat serum in PBS at 4°C overnight. After washing, the samples were incubated with secondary antibodies (1: 2500, FITC-conjugated goat anti-mouse IgG and Texas Red-conjugated goat anti-rabbit IgG, SantaCruz Biotechnology) at room temperature for 1 hour. After rinsing, glass coverslips were mounted with Vector Mounting medium and cells were examined with a Nikon microscope equipped with filter cubes for the detection of FITC and Texas Red fluorescence. The number of cells

that express loricrin and/or HA-C/EBPα was counted directly from 100 randomly chosen fields.

RESULTS AND DISCUSSION

C/EBPα protein and mRNA levels are greatly diminished in SCC cell lines

We have previously shown that C/EBPα and C/EBPβ are expressed abundantly in mouse keratinocytes (16). In order to determine if C/EBPα expression is altered in SCCs, we first examined C/EBPα protein levels in seven mouse SCC cell lines. These SCC cell lines were isolated from various mouse skin SCCs which were generated by treatment with 7, 12-dimethylbenz[a]anthracene (DMBA) and then promoted with mirex and/or 12-O-tetradecanoylphorbol-13-acetate (TPA). All of SCC cell lines contain oncogenic H-Ras mutations in the 61st codon (data not shown). As shown in Fig. 1A, the C/EBPα protein levels were greatly diminished in all seven SCC cell lines compared to normal keratinocytes. In contrast to C/EBPα protein levels, C/EBPβ protein levels were not dramatically changed compared to normal keratinocytes. To determine whether this decrease in C/EBPα protein level is associated with decreased mRNA expression, we examined C/EBPα mRNA levels in the SCC cell lines. As shown in Fig. 1B, C/EBPα mRNA levels in SCC cell lines were also greatly diminished compared to normal keratinocytes. In general, the level of C/EBPα mRNA expression was proportional to the protein levels suggesting that the decrease in C/EBPα protein in SCC cell lines is likely due to decreased levels of C/EBPα mRNA. Our findings that C/EBPα mRNA/protein levels are greatly decreased in skin SCC cell lines is in accord with reports that C/EBPα levels are decreased in hepatocellular carcinomas and lung cancer cell lines (14, 15).

Treatment with cytosine methylation inhibitor induces C/EBPα expression in SCC cell lines.

While down-regulation of C/EBPα mRNA in SCC cell lines is evident, it is not clear

what mechanisms are responsible. Epigenetic mechanisms, such as promoter hypermethylation, are associated with transcriptional silencing of several tumor suppressor genes such as Rb (20, 21), $p16^{INK4a}$ (22-24), E-cadherin (25), Brca1 (26), and PTEN (27). 5-aza-2'-deoxycytidine (5-aza-dC) is an inhibitor of cytosine methylation and is known to induce re-expression of gene inactivated by promoter hypermethylation (25, 28). In addition, Cameron et al (29) showed there is a synergistic effect of co-treatment of 5-aza-dC and trichostatin A (TSA), a histone deacetylase inhibitor, on the re-expression of genes silenced by hypermethylation in cancer. We treated SCC cells with 5-aza-dC to determine if this treatment could alter the expression of C/EBPα. SCC cell lines were treated with 5-aza-dC for 72 hours and the level of C/EBPα protein was analyzed by western blot. C/EBPα protein level was increased after 5-aza-dC treatment in 4/7 SCC cell lines (Fig. 2A). Furthermore, co-treatment of SCC cell lines with 5-aza-dC and TSA resulted in synergistic increase in re-expression of C/EBPα protein in 2/7 SCC cell lines (Fig. 2B). These results suggest that C/EBPα or another transcription factor that regulates C/EBPα expression may be transcriptionally silenced by promoter hypermethylation.

Forced expression of C/EBPα induces growth arrest in SCC cell lines

We previously have shown that forced expression of C/EBPα induced growth arrest and differentiation-like morphological changes in keratinocytes (17). In order to examine the effect of C/EBPα on growth of SCC cell lines, we transfected SCC cell lines with pcDNA3-C/EBPα. Transfected SCC cells were selected in the presence of G418 and the number of colonies and the number of cells per colony were counted during the selection period. As shown in Fig. 3A, 3B, and 3C, the number of colonies in C/EBPα transfected SCC cell lines was greatly reduced compared to pcDNA3-

transfected SCC cells. The number of cells per colony was also decreased in the C/EBPα transfected cells compared to pcDNA3 transfected cells. Moreover, during the selection period the number of cells/colony in C/EBPα transfected SCC cells increased very slowly compared to the empty vector transfected cells. C/EBPα-induced growth arrest was observed in other SCC cell lines (data not shown). These results indicate that C/EBPα inhibits the proliferation of SCC cells.

Forced expression of C/EBPα induces expression of loricrin

We have previously shown that C/EBPβ has a role in early squamous differentiation of keratinocytes as the expression of keratin 1 (K1) and keratin 10 (K10), two early markers of squamous differentiation, are reduced in C/EBPβ deficient keratinocytes (17). In addition, the forced expression of C/EBPβ, but not C/EBPα induced the expression of K1 and K10 in BALB/MK2 keratinocytes. However, the role of C/EBPα in other differentiation events involving keratinocytes has not been investigated, although Agarwal et al (30) recently showed C/EBPα increases human involucrin promoter activity. In order to study the effect of C/EBPα on keratinocyte differentiation, we transfected BALB/MK2 cells with HA-tagged C/EBPα and performed double-immunofluorescence staining to examine if C/EBPα induces the expression of differentiation markers in BALB/MK2 keratinocytes. BALB/MK2 keratinocytes are a non-transformed, immortalized mouse keratinocyte cell line which undergoes differentiation in response to changes in calcium concentrations in the medium (31) and displays intermediate expression of C/EBPα (data not shown). As previously reported, forced expression of C/EBPα did not induce expression of K10, however, C/EBPα expression did induce the expression of loricrin, a late stage marker of squamous differentiation (32). We observed that 80 % of loricrin positive stained

cells also expressed HA-tagged C/EBPα (Fig. 4A and B). This co-localization of C/EBPα and loricrin suggests that C/EBPα induces the expression of loricrin. Thus, it appears that C/EBPα has effects on both proliferation and differentiation of keratinocytes.

C/EBPα expression is down-regulated in SCC

To determine whether the decrease in C/EBPα expression observed in the SCC cell lines also occurs in the primary cancer lesion itself, the immunohistochemical staining for C/EBPα in mouse skin SCCs was conducted. We examined 14 SCCs and 2 keartoacanthomas that were generated by DMBA/TPA initiation-promotion protocol. As shown in Fig. 4C, there is strong nuclear C/EBPα staining in suprabasal layer of epidermis where post-mitotic keratinocytes undergo differentiation and weak perinuclear staining of C/EBPα in basal layer. All 14/14 SCCs demonstrated little to no C/EBPα immunostaining as did the two keratoacanthomas (data not shown). A representative micrograph of a SCC is shown in Fig. 4E with intact epidermis above the tumor for comparison of C/EBPα immunostaining. A higher power micrograph of the SCC is shown in Fig. 4G. These data confirm the decrease in C/EBPα levels in the 7 SCC cell lines is also present in the primary tumor itself. In contrast to C/EBPα, immunostaining for C/EBPβ was detected in the SCCs (Fig. 4D, F, and H). Collectively, our results suggest that the loss of C/EBPα expression contributes to the altered growth and differentiation characteristics of skin SCCs. Our results contribute the emerging evidence that C/EBPα may have a tumor suppressor-like function in numerous types of cancer.

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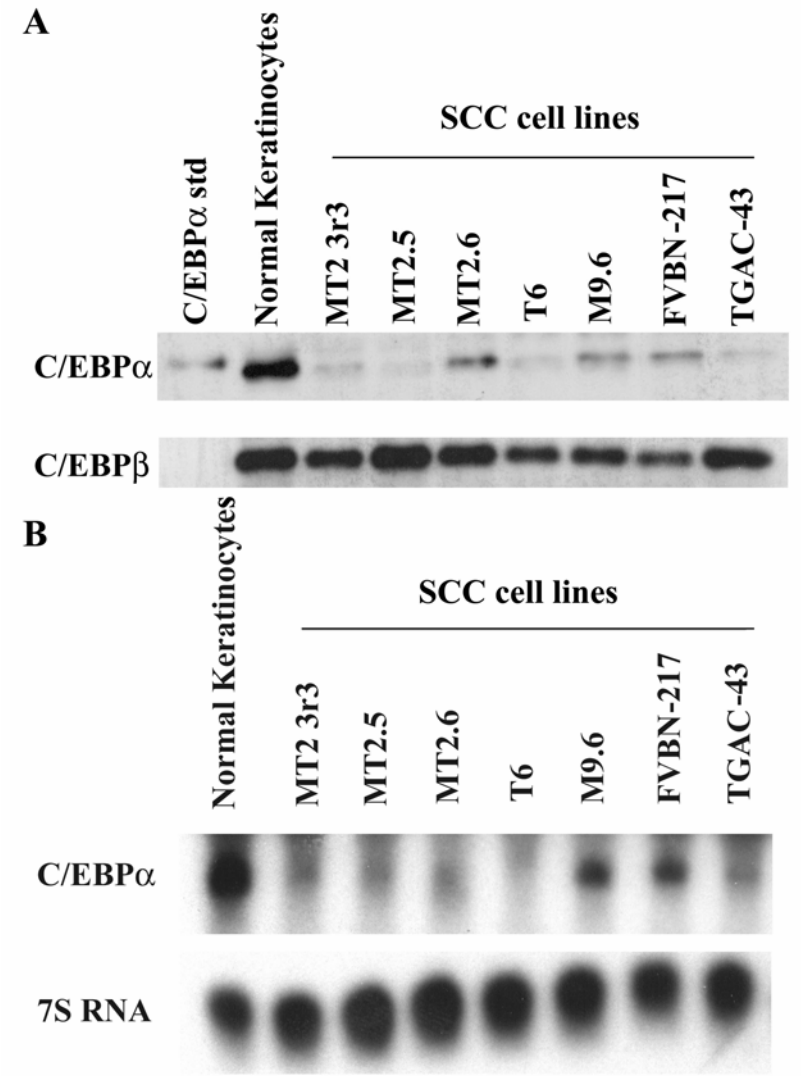


Fig. 1 *C/EBPα protein and mRNA levels are greatly diminished in SCC cell lines.*

A) Nuclear extracts were prepared from normal mouse keratinocytes and SCC cell lines and equal amounts of protein (20 μg) were subjected to western blot analysis using rabbit polyclonal anti-C/EBPα or anti-C/EBPβ antibody. C/EBPα protein standard was histidine-tagged and runs slightly slower than non-tagged C/EBPα. B) Total cellular RNA was isolated from normal keratinocytes and SCC cell lines and northern blot analysis was conducted. The membrane was reprobed with 7S RNA cDNA to verify the equal loading of RNA.

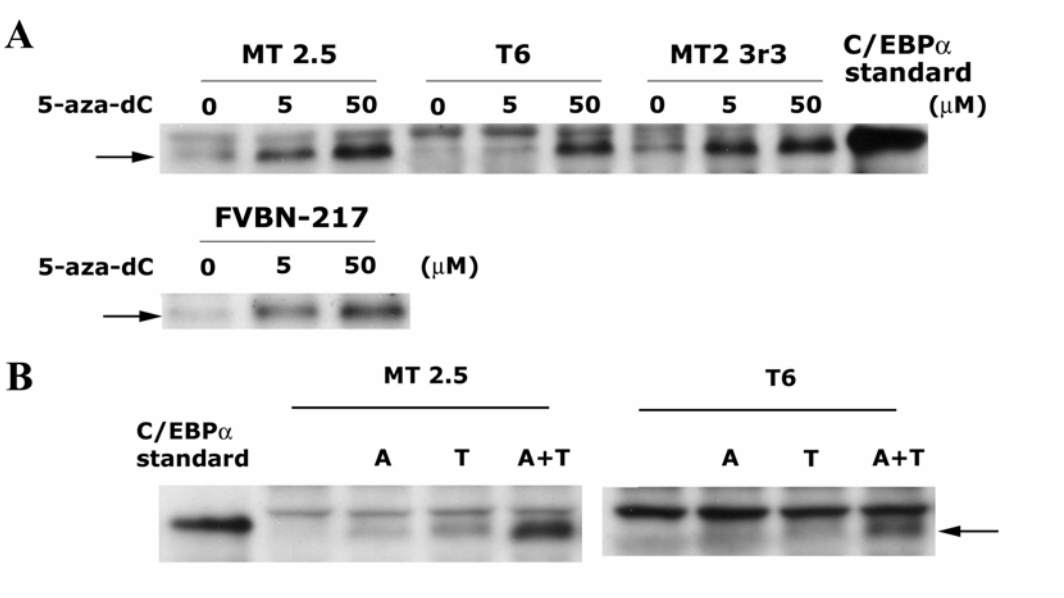
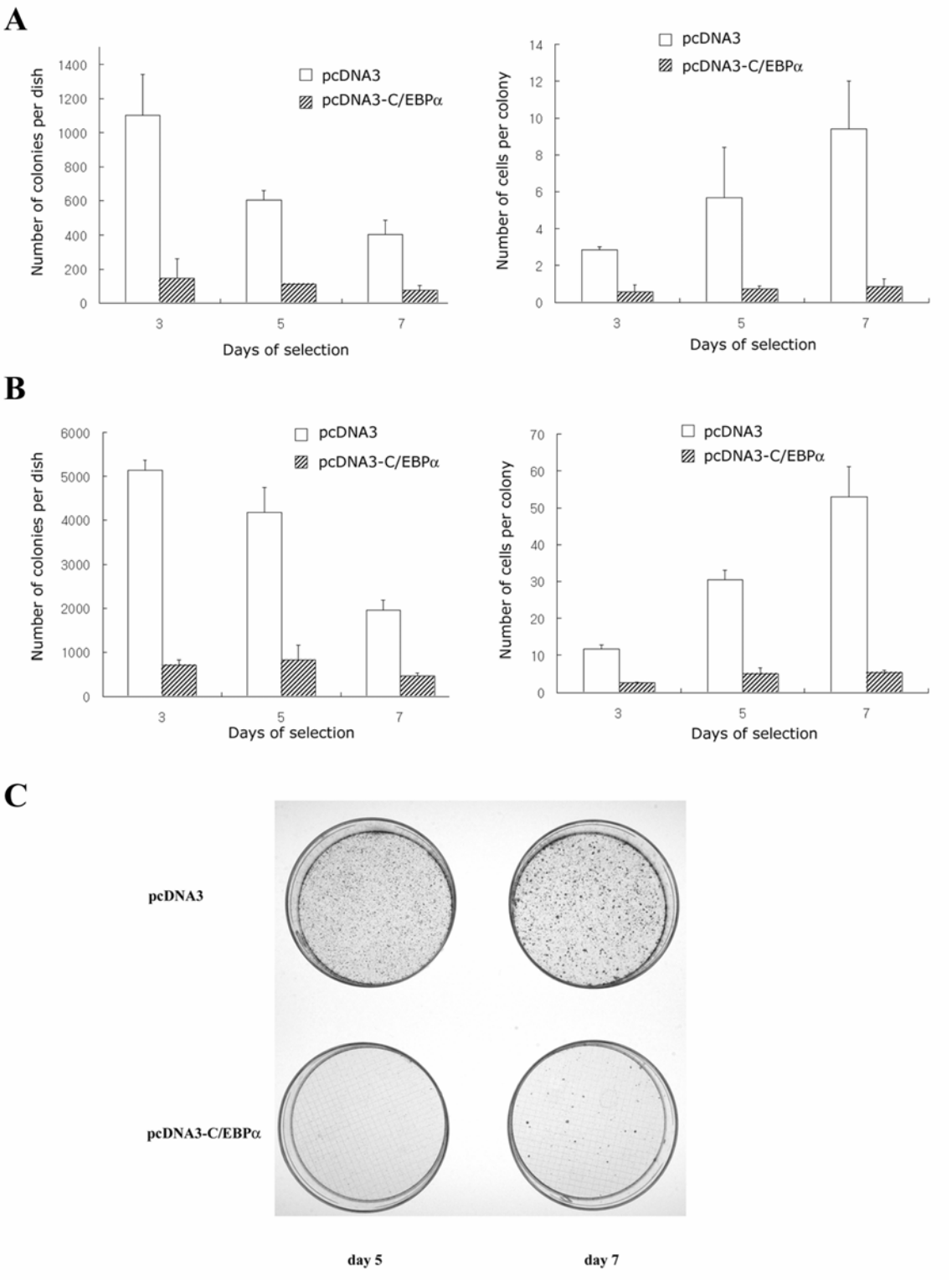


Fig. 2 *Treatment with methylation inhibitor induces C/EBPα expression in SCC cell lines.*

A) MT2.5, MT2 3r3, T6, and FVBN-217 mouse skin SCC cell lines were treated with specified concentrations of 5-aza-2'-deoxycytidine for 72 hours. Whole lysates were prepared and equal amounts of protein (40 μg) were subjected to western blot analysis using rabbit polyclonal anti-C/EBPα antibody. Arrow indicates C/EBPα. B) MT2.5 and T6 SCC cell lines were treated with 5-aza-2'-deoxycytidine and trichostatin A as described in Materials and Methods. Whole lysates were prepared and equal amounts of protein (40 μg) were subjected to western blot analysis using rabbit polyclonal anti-C/EBPα antibody. Arrow indicates C/EBPα.


A
Number of colonies per dish
1400
1200
1000
800
600
400
200
0
pcDNA3
pcDNA3-C/EBPα
3
5
7
Days of selection
Number of cells per colony
14
12
10
8
6
4
2
0
pcDNA3
pcDNA3-C/EBPα
3
5
7
Days of selection
B
Number of colonies per dish
6000
5000
4000
3000
2000
1000
0
pcDNA3
pcDNA3-C/EBPα
3
5
7
Days of selection
Number of cells per colony
70
60
50
40
30
20
10
0
pcDNA3
pcDNA3-C/EBPα
3
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Days of selection
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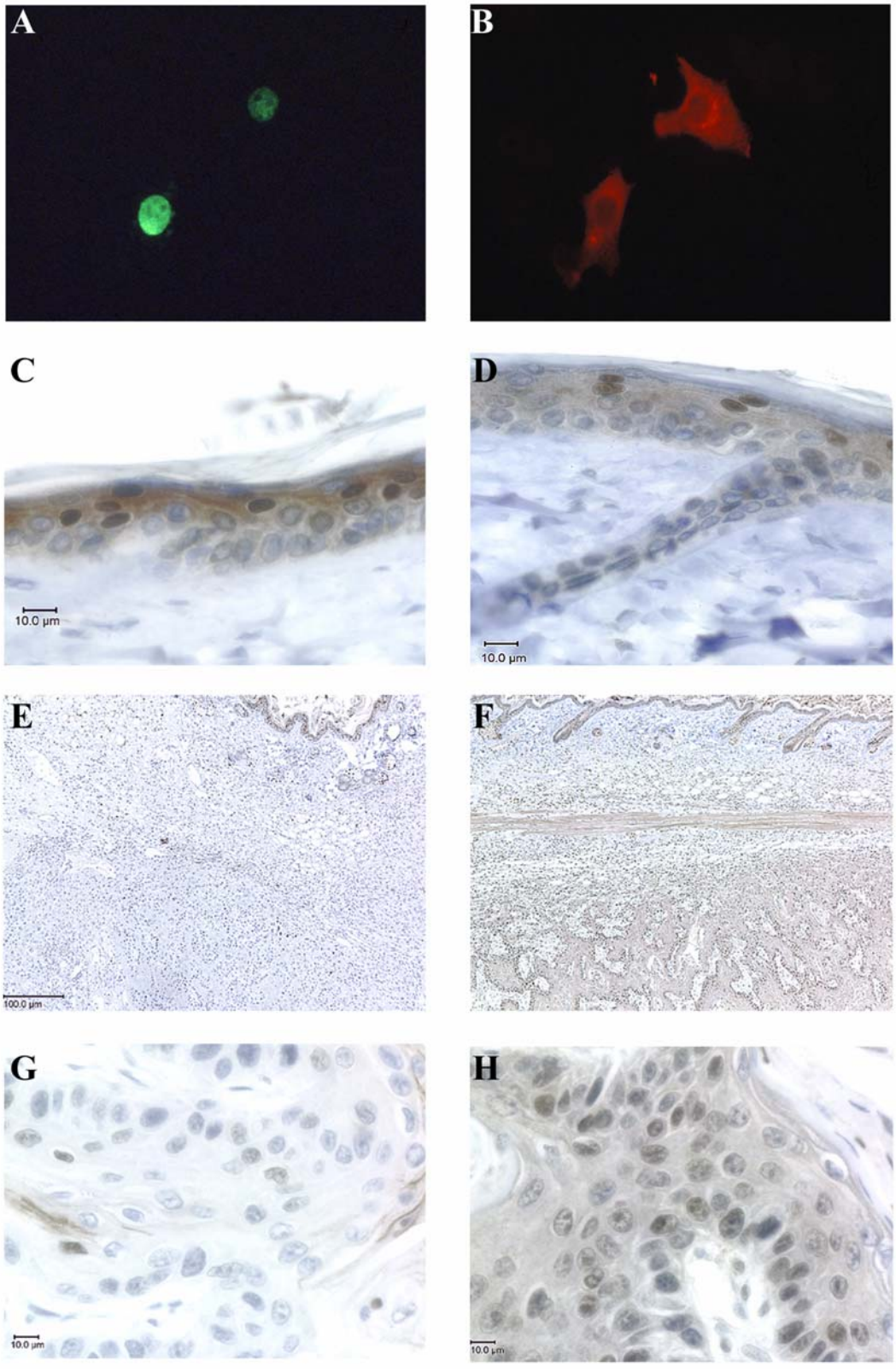
pcDNA3
pcDNA3-C/EBPα
day 5
day 7

Fig.3 *Forced expression of C/EBPα induces growth arrest in SCC cell lines.*

A) MT2.5 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subsequently subcultured in the presence of 300 μg/ml G418. The number of colonies per dish (left panel) and the number of cells per colony (right panel) were determined at days 3, 5, and 7 of G418 selection. Data are expressed as the mean +/- standard deviation of a representative experiment done in triplicate. B) MT2 3r3 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subsequently subcultured in the presence of 300 μg/ml G418. The number of colonies per dish (left panel) and the number of cells per colony (right panel) were determined at days 3, 5, and 7 of G418 selection. Data are expressed as the mean +/- standard deviation of a representive experiment done in triplicate. C) MT2 3r3 SCC cells were transfected with pcDNA3 or pcDNA3-C/EBPα and subcultured in the presence of 300 μg/ml G418. On days 5 and 7 of G418 selection, the cells were stained with crystal violet and photographs of colonies were taken.


A
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C
10.0 µm
D
10.0 µm
E
100.0 µm
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G
10.0 µm
H
10.0 µm

Fig. 4 *Forced expression of C/EBPα induces expression of loricrin and C/EBPα expression is down-regulated in SCCs.*

A, B) Immunofluorescence detection of the co-expression of C/EBPα and loricrin in pCMV-HA-C/EBPα transfected BALB/MK2 keratinocytes. BALB/MK2 keratinocytes were transiently transfected with pCMV-HA-C/EBPα and processed for detection of HA (FITC) and loricirn (Texas Red) coexpression. C) C/EBPα immunostaining in normal epidermis (higher magnification). D) C/EBPβ immunostaining in normal epidermis (higher magnification). E) C/EBPα immunostaining in SCC with epidermis above the tumor (lower magnification). F) C/EBPβ immunostaining in SCC with epidermis above the tumor (lower magnification). G) C/EBPα immunostaining in SCC (higher magnification). H) C/EBPβ immunostaining in SCC (higher magnification).

GENERAL DISCUSSION

Normal epidermis is a self-renewing tissue that maintains homeostasis via precise control of proliferation and differentiation. Epidermal homeostasis is maintained by a number of cellular signaling pathways, however, failure in the regulation of epidermal homeostasis results in skin disorders such as psoriasis and skin cancer. Therefore, understanding the mechanisms that regulate epidermal homeostasis is important for providing better diagnosis and preventive/therapeutic approaches for skin cancer as well as other skin diseases.

C/EBPα is a transcription factor that is associated in a variety of cellular processes, such as proliferation, differentiation, and metabolism (28, 74, 77, 209-211) and has been shown to be abundantly expressed in mouse keratinocytes (29). The transactivation activity of C/EBPα can be regulated in several ways, including transcriptional, translational, and post-transcriptional control. However, little is known about the mechanism of C/EBPα turnover. Properly regulated degradation of C/EBPα would be critical in C/EBPα-mediated cellular processes in which the rates of synthesis and destruction of C/EBPα needs to be balanced. The ubiquitin/proteasomal degradation system plays an important role in the degradation of cellular proteins which are involved in regulating various cellular process, including differentiation, cell cycle, or apoptosis (136, 137, 151, 178).

In manuscript I, we demonstrated that C/EBPα is degraded via proteasome in an ATP- and ubiquitin dependent manner and lithium, a known inhibitor of GSK3 (212),

regulates this process by inhibiting proteasomal degradation of C/EBPα in a GSK3 independent manner. The pathway for ubiquitin conjugation reaction is well characterized in which ubiquitin molecule is transferred to the target molecule by sequential action of E1, E2, and E3 ligase. Exactly how proteins are selected for their degradation is beginning to be clarified and degradation of substrates by ubiquitin/proteasome system appears to be mediated by specific degradation signals such as phosphorylation. Identification of a signal for ubiquitination of C/EBPα and E3 ligase which is responsible for proteasomal targeting of C/EBPα will require further studies. Since C/EBPα levels are highest in terminally differentiated cells (e.g. adipocytes) and down-regulated after partial hepatectomy (proliferation) (28), C/EBPα levels may be regulated via proteasomal degradation in a cell cycle dependent manner. Kumatori et al reported that proteasome is expressed at higher levels in leukemia cells than in normal cells and proteasome expression is increased in parallel with induction of DNA synthesis (213), suggesting high levels of proteasome expression is associated with proliferation or transformation. We observed that C/EBPα mRNA and protein levels are very low in SCC cell lines however, we do not know whether these SCC cell lines have higher proteasomal activity than normal keratinocytes. Since C/EBPα transcription can be subjected to autoregulation (59, 60), an increase in the rate of C/EBPα degradation may affect its mRNA levels.

In manuscript II, we demonstrated that C/EBPα expression levels are greatly diminished in mouse SCC / SCC cell lines and C/EBPα negatively regulates SCC cell proliferation suggesting that loss of C/EBPα is an important event in SCC development.

While it is clear that C/EBPα negatively regulates proliferation of SCC cells, it is also possible that C/EBPα induces differentiation or initiates apoptotic response in SCC cell lines, thereby inhibiting cell proliferation. Preliminary evidence indicates that C/EBPα is associated with differentiation of keratinocytes. However, in order to identify the mechanism underlying C/EBPα-mediated growth arrest, development of inducible cell line is required. In addition, whether human SCC or SCC cell lines also have decreased levels of C/EBPα mRNA/proteins requires further studies.

It has been suggested that there are multiple pathways by which C/EBPα induces growth arrest. For example, C/EBPα directly represses E2F dependent transcription (214) and Johansen et al have found that C/EBPα represses c-myc expression in myeloid cells through an E2F binding site in c-myc promoter (215). It also has been shown that C/EBPα interacts with components of the cell cycle machinery such as Rb, p107, and p21 (98, 216). In addition, a recent study suggested that C/EBPα directly interacts with cdk (cyclin dependent kinase) 2 and cdk4 and arrest cell proliferation by inhibiting these kinases (100). Further studies are required to identify the molecular mechanism by which C/EBPα induces growth arrest in SCC cell lines.

Disruption of tumor suppressor functions by inactivating mutations is one of the common mechanisms in human cancer. However, hypermethylation of CpG islands in promoter region of tumor suppressor genes represents an alternative mechanism to inactivate tumor suppressor functions (217-219). Hypermethylation of CpG island is an epigenetic event that is not accompanied by changes in DNA sequence. Recent evidence supports the notion that the promoter hypermethylation of tumor

suppressor genes plays a major role in carcinogenesis by silencing key cancer-related genes such as Rb (220, 221), $p16^{INK4a}$(222-225), E-cadherin (226), Brca1 (227), and PTEN (228). Recently, hypermethylation of C/EBPα promoter in a certain type of human acute myeloid leukemia was reported (229) although the incidence of hypermethylation in C/EBPα promoter was infrequent (two out of seventy). We have shown that 5-aza-2'-deoxycytidine, a demethylating agent (230-232), induces C/EBPα expression in SCC cell lines. Furthermore, histone deacetylase inhibitor synergized with demethylating agent for re-expression of C/EBPα in SCC cell lines (233). The C/EBPα promoter region has several stretches of CG-rich sequences and further studies are required to identify specific CpG residues which are methylated.

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APPENDIX

C/EBPβ Modulates the Early Events of Keratinocyte Differentiation Involving Growth Arrest, Keratin 1 and Keratin 10 Expression

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Abbreviations: BrdU, bromodeoxyuridine; C/EBP, CCAAT/enhancer binding protein; MGF, myelomonocytic growth factor; K1, keratin 1; K5; keratin 5; keratin 10, K10

ABSTRACT

The epidermis is a stratified squamous epithelium composed primarily of keratinocytes that become postmitotic and undergo sequential changes in gene expression during terminal differentiation. The expression of the transcription factor CCAAT/enhancer binding protein-β (C/EBPβ) within mouse epidermis and primary keratinocytes has recently been described; however, the function of C/EBPβ within the epidermis/keratinocyte is unknown. We report here that transient transfection of mouse primary keratinocytes with a C/EBP responsive promoter/reporter construct resulted in a 7-fold increase in luciferase activity when keratinocytes were switched to culture conditions that induce growth arrest and differentiation. Forced expression of C/EBPβ in BALB/MK2 keratinocytes inhibited growth, induced morphological changes consistent with a more differentiated phenotype and upregulated two early markers of differentiation, keratin 1 (K1) and keratin 10 (K10) but had a minimal effect on the expression of late stage markers, loricrin and involucrin. Analysis of the epidermis of C/EBPβ deficient mice revealed a mild epidermal hyperplasia and decreased expression of K1 and K10 but not involucrin and loricrin. C/EBPβ deficient primary keratinocytes were partially resistant to calcium induced growth arrest. Analysis of terminally differentiated spontaneously detached keratinocytes or those induced to differentiate by suspension culture revealed that C/EBPβ deficient keratinocytes displayed striking decreases in K1 and K10, while expression of later stage markers were only minimally altered. Our results demonstrate that C/EBPβ plays an important role in the early events of stratified squamous differentiation in keratinocytes involving growth arrest and K1 and K10 expression.

INTRODUCTION

The epidermis is a stratified squamous epithelium composed primarily of keratinocytes that form four distinct morphological layers. Each epidermal layer or compartment represents a different phenotypic stage in the terminal differentiation program of the keratinocyte. This program begins when the basal keratinocyte becomes postmitotic and initiates its migration upward through the spinous and granular layers to eventually form the nonviable cornified stratum corneum (for reviews 18, 52). The process of stratified squamous differentiation is a dynamic one involving a highly coordinated program of gene expression that includes both induction and repression. For example, the transition of the basal keratinocyte from the basal layer to spinous layer is accompanied by the repression of basal keratinocyte transcripts keratin 5 (K5), keratin 14 (K14) (17, 56) and α4β6 integrin (49) and the upregulation of the early stage differentiation markers, keratin 1 (K1) and keratin 10 (K10) (34, 37, 41). The transition from the spinous to granular layer is accompanied by the suppression of K1 and K10 transcripts and the up-regulation of transcripts for the cornified envelope precursor proteins such as involucrin, loricrin and filaggrin (13, 14, 15, 26, 39). Epidermal transglutaminase crosslinks these and other proteins to form the cornified envelope and subsequent to the digestion of the intracelluar organelles the mature nonviable squame is formed. While the stages of squamous differentiation with their concomitant changes in gene expression are well characterized, the transcription factors that regulate the induction and repression of differentiation-specific genes remain largely uncharacterized.

The C/EBP family of transcription factors is composed of at least five distinct members [C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, Ig/EBP(C/EBPγ)] (6, 55, for review

54) belonging to the basic leucine zipper (bZIP) class of transcription factors. C/EBPα and C/EBPβ are expressed in human and mouse primary keratinocytes (31, 50) as well as in the human, mouse and rat interfollicular epidermis (25, 31, 47). Within the mouse interfollicular epidermis, C/EBPα is expressed in the nuclei and cytoplasm of suprabasal keratinocytes and weakly expressed in a perinuclear manner in some basal keratinocytes (31). C/EBPβ expression is highly compartmentalized and is exclusive to the nuclei of a 3-cell cluster of suprabasal keratinocytes which is morphologically consistent with the differentiative column of the epidermal proliferative unit (EPU). In primary mouse keratinocytes, C/EBPβ expression is upregulated during calcium-induced growth arrest and squamous differentiation (31). Thus, C/EBPβ appears to have a role in the regulation of genes involved in or specifically expressed during squamous differentiation of the epidermis. Additional indirect evidence for a role for C/EBPβ in squamous differentiation comes from the observations that C/EBPβ expression is greatly diminished in squamous cell carcinomas (31) as is the expression of keratin 1 (K1), keratin 10 (K10), loricrin and filaggrin (59).

C/EBPβ (also known as NF-IL6, IL-6DBP, NF-M, CRP2, or LAP) is involved in the regulation of the expression of a number of cytokine genes and C/EBPβ binding motifs are found in the regulatory regions of IL-1β, IL-6, IL-8, TNFα, and granulocyte-colony stimulating factor (G-CSF) (1, 11, 28, 29, 60). C/EBPβ also plays a role in the early stages of preadipocyte differentiation (6, 57) and differentiation of certain cells of the myeloid lineage (29, 42). C/EBPβ-deficient mice display immune defects including lymphoproliferative disorder, distorted humoral, innate and cellular immunity, and imbalanced T-helper cell response (43) and impaired tumor cytotoxicity and bactericidal activity of macrophages (48). Female mice lacking C/EBPβ are infertile

due to the failure of ovarian granulosa cells to differentiate into luteal cells (46) and these mice also demonstrate defects in the proliferation and differentiation of mammary epithelial cells (36, 44).

In the present study we have evaluated the role of C/EBPβ in epidermal keratinocyte proliferation and squamous differentiation. We have examined the transactivation activity of endogenous C/EBP in primary keratinocytes under both proliferative and differentiative conditions and have evaluated the effect of the forced expression of C/EBPβ on keratinocyte growth and differentiation. In addition, we have analyzed the epidermis of C/EBPβ deficient mice in vivo and have isolated primary keratinocytes from these mice and examined their ability to undergo growth arrest and terminal differentiation. Our results demonstrate that C/EBPβ plays an important role in the early stages of squamous differentiation involving growth arrest and K1 and K10 expression.

MATERIALS AND METHODS

Materials

Fetal bovine serum, trypsin, antibiotics/antimycotics, and protein molecular weight markers were purchased from GIBCO BRL (Gaithersburg, MD). EMEM (Ca^{++} free) was purchased from BioWhittaker (Walkersville, MD). Human recombinant epidermal growth factor (hEGF) was purchased from United States Biochemical (Cleveland, OH). pcDNA3 expression vector and the PerFect Lipid, Pfx-3 were purchased from Invitrogen (San Diego, CA). Rabbit polyclonal antibodies for C/EBPα, C/EBPβ and $p21^{Cip1/WAF1}$ and mouse monoclonal antibody to C/EBPβ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Keratin 1, keratin 10, keratin 5, and involucrin rabbit polyclonal antibodies were purchased from Berkeley Antibody Company (Richmond, CA). Rabbit polyclonal antibody for loricrin was a kind gift from Dr. G. Paolo Dotto, Harvard Medical School, Charlestown, MA. Mouse monoclonal BrdU antibody was purchased from Becton Dickinson (San Jose, CA). Goat anti-rabbit IgG Texas Red and goat anti-mouse IgG FITC were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Horseradish peroxidase-linked donkey anti-rabbit IgG and ECL kit were purchased from Amersham (Arlington Heights, IL). 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). [^{3}H-methyl]-Thymidine (20 Ci/mmol) was purchased from DuPont-New England Nuclear Research Products (Boston, MA). Bromodeoxyuridine (BrdU), methylcellulose (4000 centipoises) and calcium chloride were purchased from Sigma (St. Louis, MO). Tris-Glycine pre-cast gels were from Novex (San Diego, CA). BioRad DC protein assay

reagent was purchased from BioRad (Richmond, CA).

Animals

CD-1 mice were purchased from Charles River Laboratory (Raleigh, NC). C/EBPβ deficient mice generated by homologous recombination have been described previously (46). C/EBPβ deficient male mice were mated with heterozygous female mice to produce greater yields of C/EBP β deficient mice. C/EBPβ +/+ mice were mated to generate control subjects. Both mutants and controls represented F2-F4 crosses of C57BL6 and 129/SV strains. Mice were genotyped by Southern analysis of tail DNA as described (46). The mice were fed # 5001 rodent chow (Purina Mills, Inc. Richmond, IN) 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.

Isolation and cultivation of primary epidermal keratinocytes

Primary keratinocytes were isolated from newborn CD-1, C/EBPβ wildtype or C/EBPβ deficient mice (less than 3 days old) by overnight trypsin floatation at $4^{o}C$ (10, 19). C/EBPβ deficient newborn mice were genotyped by Western analysis using whole liver homogenates. Isolated keratinocytes (pooled from 5-10 newborn mice) were plated at 6×10^6 cells/60 mm plate, or 0.75×10^6 cells/well in 24 well culture dishes in Ca^{++}-free EMEM medium supplemented with 10% non-chelex treated fetal bovine serum and 4 ng/ml hEGF for 4 hours to enhance cell attachment. Cultures were then gently washed with Mg^{++} and Ca^{++} free PBS to remove any remaining calcium and unattached cells, and then refed with low calcium medium (Ca^{++} free EMEM supplemented with 4% chelex-treated fetal bovine serum, 10 ng/ml hEGF, 100 u/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml fungizone/ml, with added calcium chloride to a final concentration of 0.05 mM). Medium was changed daily.

Transfection of primary CD-1 keratinocytes and luciferase assays

Primary CD-1 keratinocytes (3 days after plating) were transfected in triplicate with the following constructs: pXP1, pMGF40, pMGF65, or pMGF82 (45). Two μg of vector DNA and 12 μg of lipid transfection reagent, pFx-3 were incubated for 20 minutes at room temperature to form complexes, and overlayed onto primary keratinocyte culture in the serum free EMEM media containing 4 ng/ml hEGF and 0.05 mM calcium chloride. Cultures were incubated at 37^{o}C, 5% CO_2 for 4 hours, then washed with PBS and refed with low calcium media. After 15 hours, cultures were either switched to high calcium medium (0.12 mM) or refed low calcium medium (0.05 mM). Forty eight hours later cells were harvested and the luciferase activity was determined using luciferase assay kit (Promega). Protein concentrations were determined using Bio-Rad DC protein assay.

Construction of C/EBPβ vector and its transfection in BALB/MK2 keratinocytes

The C/EBPβ coding region (~0.8 kb) containing a Kozak translation initiation sequence was released from pMEX- C/EBPβ vector (55) by *BamHI* and *KpnI* digestion, and ligated to linearized pcDNA3 (by *BamHI* and *EcoRI*) at the *BamH1* site. This ligated vector was recut by *KpnI* (pcDNA3 contains a *KpnI* site 5' to *BamHI* site) to release the coding sequence of C/EBPβ with *KpnI* sites on both ends, which was used as the insert DNA in a final ligation reaction with *Kpn1*-linearized pcDNA3. The resulting ligated vector was transformed into One-Shot Top10F'competent cells (Invitrogen, Carlsbad, CA), and vector DNA was prepared from expanded individual colonies. The recombinant vector containing a single copy of C/EBPβ insert (determined by restriction enzyme mapping analysis) in sense orientation (determined by PCR analysis) was designated as pcDNA3-C/EBPβ.

BALB/MK2 keratinocytes were a gift from Dr. B. Weissman (University of North Carolina, Chapel Hill, NC). BALB/MK-2 keratinocytes were transfected when they reached 30-40% confluence in 60 mm dishes with 2 μg of vector DNA (pcDNA3 or pcDNA3- C/EBPβ) and 12 μg of lipofection reagent, pFx-3. Transfection was performed in serum free EMEM media (containing 0.05 mM calcium and 4 ng/ml hEGF) at 37°C, 5% CO_2 for 15 hours, after which time the cells were refed with low calcium medium (Ca^{++} free EMEM supplemented with 8% chelex-treated FBS, 4 ng/ml hEGF and calcium chloride to a final concentration of 0.05 mM). Twenty four hours later the cultures were split (1:5) and replated in the above medium. Twenty four hours after replating, G418 was added to the medium at a concentration of 500 μg/ml, and this selection medium was changed every other day. On days 3, 5, 7 and 10 after G418 selection, the total number of colonies in 50 random grid squares were counted and then converted to colonies/dish (550 grid squares/plate). The number of cells per colony was scored directly from 50 randomly chosen colonies.

Immunochemical staining of C/EBPβ, involucrin, loricrin, keratin 1 and keratin 10 in pcDNA3 and pcDNA3-C/EBPβ transfected BALB/MK2 keratinocytes

BALB/MK2 cells were transfected by pcDNA3 and pcDNA3-C/EBPβ vector as described in previous section. Forty eight hours after transfection cultures were rinsed 3 times with PBS and fixed in cold methanol for 10 minutes. The endogenous peroxidase activity was quenched by incubation in 0.1% H_2O_2 in PBS for 10 minutes at room temperature. After 3 washings with PBS, the cultures were blocked with 1.5% normal goat serum (NGS) in PBS for 30 minutes at room temperature and then incubated with the primary rabbit polyclonal antibodies against C/EBPβ, K1, K10, involucrin or loricrin (all 1:2000) in 1.5% NGS in PBS at 4°C overnight. After 3

washings with PBS, the samples were incubated with a biotinylated goat-anti-rabbit IgG for 30 minutes at room temperature followed by a 30 minutes incubation with peroxidase conjugated streptavidin. The avidin/biotin-peroxidase complexes were visualized by incubation with 5, 5'-diaminobenzidine following the manufacturer's protocol. Cultures incubated with the secondary antibody alone (biotinylated goat-anti-rabbit IgG) did not develop any positive immunostaining. Cultures were observed at 100X magnification and single dark-brown stained positive cells were counted in 25 fields per sample. Results are expressed as the number of positive cells per field. Observations from 10 fields/sample showed that there was no significant difference in the total number of cells/field in pcDNA3 vector control (2280 ± 190) and pcDNA3-C/EBPβ transfected cultures (2250 ± 210). For immunofluorescence dectection of C/EBPβ and K1; BALB/MK2 cells were plated onto coverslips (0.5 inch diameter) in 60 mm dishes and transfected as described above. Forty eight hours after transfection the cultures were fixed in methanol at -20˚C for 10 min, and coverslips were mounted on a microscope slide. Coverslip cultures were treated as described above and then incubated with rabbit K1 polyclonal antibody (1:2000) and mouse C/EBPβ monoclonal antibody (1:2000) in 1.5% NGS at 4˚C overnight. After washing, the samples were incubated with secondary antibodies (FITC-conjugated goat anti mouse IgG and Texas Red conjugated goat anti rabbit IgG) at room temperature for 30 minutes. After rinsing, glass coverslips were mounted over the samples with Vector Mounting medium and cells were examined using a Nikon microscope equipped with filter cubes for the detection of FITC and Texas Red fluorescence.

Western analysis of C/EBPs and various differentiation-associated marker proteins

Pooled primary keratinocytes isolated from newborn wildtype and C/EBPβ deficient

mice were grown in low calcium medium with medium change daily. On day 5, one set of the cultures was detached from the plates by trypsinization and inoculated into a suspension culture medium (low calcium medium plus 1.4% methylcellulose) at a density of 2 x 10^6 cells/ml and incubated at 37°C, 5% CO_2 for 16 hours. On day 6 attached cells, spontaneously detached cells and suspension cultured cells were harvested separately, and placed in a lysis buffer (10 mM Tris HCl, pH 7.5 containing 5% SDS and 20% β-mercaptoethanol). Cell lysates were sonicated for 5 seconds and boiled for 5 minutes. For the in vivo study, protein samples were prepared from epidermis of both wildtype and C/EBPβ deficient female mice (16-18 weeks old). Dorsal hair was clipped with electric clippers, dorsal skin removed and epidermal cells were isolated by trypsin floatation (10, 19). The isolated cells were placed directly into the above lysis buffer, sonicated, centrifuged to remove hair fibers, and then boiled for 5 minutes. In order to determine the protein concentration, a portion of each sample was first precipitated in 6% trichloroacetic acid in the presence of 125 μg/ml of Na-deoxycholate (4), and then quantitated by Lowry assay (23). Equal amount of each protein samples were loaded on 10 or 12% polyacrylamine Tris-Glycine gels (Novex) and separated by electrophoresis. The separated proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA). Following incubation in blocking buffer (PBS with 1% BSA, 5% milk, and 0.1% Tween) for 1 hour at room temperature, the membranes were probed overnight at 4°C with rabbit polyclonal IgG raised against C/EBPα (1:2,000), C/EBPβ (1:2,000), K1 (1:2000), K10 (1:2000), K5 (1:2000), involucrin (1:2000), loricrin (1:2000), or $p21^{Cip1/WAF1}$ (1:1000). The membranes were washed and then probed with a secondary antibody (1:2500 horseradish peroxidase-linked donkey anti rabbit immunoglobulin from Amersham, Arlington Heights, IL) for 1

hour at room temperature. Detection was made with an enhanced chemiluminescence reagent followed by exposure film. The densitometric quantitation of the bands of interest was conducted using a Zeineh laser scanning densitometer (Model SLR-1D/2D, Fullerton, CA).

Northern analysis of K1 expression

Primary keratinocytes isolated from wildtype and C/EBPβ deficient newborn mice were cultured in low calcium medium for seven days and the attached proliferative population of cells were collected and RNA isolated. In addition, differentiation was induced in attached keratinocytes by placing these cells in suspension culture (12) for 16 hours and these cells were also collected on day seven. RNA was isolated and northern blot analysis was conducted as previously described (31) using a ^{32}P-labeled 400 bp K1 probe (kindly provided by Dr. Stuart Yuspa, NCI, Bethesda, MD)

Analysis of epidermal keratinocyte proliferation in C/EBPβ deficient mice in vivo and in BALB/MK2 keratinocytes.

Skin histological sections were prepared from both wildtype and C/EBPβ deficient mice (24 weeks old) following our previous methods (30). In vivo BrdU labeling was conducted by a single dose i.p. injection of BrdU (100 mg/kg body weight) 1 hour before the animals were sacrificed. Immunochemical staining of BrdU positive cells was performed as described before (30). The BrdU labeling index (quantitated in 1000 interfollicular basal keratinocytes per section), the thickness of epidermis and the number of nucleated cell layers (determined in 20 locations per section) were quantitated. For BrdU labeling studies in BALB/MK2 cells; BALB/MK2 keratinocytes were transfected with pcDNA3-C/EBPβ, or empty vector control. Transfected keratinocytes were selected for 24 hrs with G418 and the number of S-

phase BrdU positive cells were determined at 0, 24 and 48 hours post G418 removal. BrdU (10 ug/ml) was added to the culture medium 4 hours before each time point and the cultures were fixed in ethanol:acetic acid (49:1) at -20˚C for 20 minutes. Immunochemical staining for BrdU was performed as described for the in vivo samples.

Primary keratinocyte proliferation determination

Pooled primary keratinocytes from newborn wildtype and C/EBPβ deficient mice were plated in 24-well culture plates as described above. The number of attached cells as well as spontaneous detached cells per well were quantitated in triplicate cultures on day 1-8 after plating. DNA synthesis was also measured every day in triplicate samples. Briefly, cultures were pulse-labeled with [^{3}H-methyl]-thymidine (3 μCi/ml) for 1 hour. After 3 washings with PBS, cells were collected by trypsinization, resuspended in 1mM EDTA buffer, sonicated for 10 seconds, and aliquot samples were collected onto glass fiber filters using a manifold sample collector. After sequential washings with cold 4% perchloric acid, 70%, 95% and 100% ethanol, the filters were counted for radioactivity in a liquid scintillation counter. DNA quantitation was conducted using Hoechst 33258-fluorometry technique (5). An aliquot of each sample and 5 μl Hoechst 33258 solution (0.1mg/ml in distilled water) were mixed in 2 ml 0.01 M Tris, pH 7.0-0.1 M NaCl-0.01 M EDTA buffer and incubated at room temperature for 5 minutes. The fluorescent units were determined using a fluorimeter (excitation at 365 nm and emmision at 450 nm). Sample DNA concentrations were determined by use of calf thymus DNA standard curve and results expressed as dpm/μg DNA. In separate experiments, the response of wildtype and C/EBPβ deficient keratinocytes to the growth inhibitory effects of calcium chloride was also studied. Primary cultures were maintained for 6-7 days in low calcium medium and switched to medium

containing 0.12 mM Ca^{++} or refed low Ca^{++} medium. [^{3}H-methyl] -Thymidine incorporation was determined as described above.

RESULTS

Endogenous C/EBP transactivation activity is increased under conditions that induce growth arrest and differentiation in primary keratinocytes

Primary mouse keratinocytes can be shifted from a proliferative state to a growth arrested state by increasing the calcium concentration in the medium from low (0.05 mM) to high (0.12 mM) (19). Following growth arrest, some keratinocytes undergo differentiation as indicated by the expression of keratin 1 (K1), keratin 10 (K10), loricrin and filaggrin (58). Previously, we have demonstrated that when primary keratinocyte cultures from CD-1 mice are switched from low to high calcium, C/EBPα protein levels are modestly increased by 20% while C/EBPβ protein level is increased 400-800% by 16 hours post calcium switch (31). In the present study, we evaluated the *trans*-activating activity of endogenous C/EBP proteins during this process utilizing a luciferase reporter gene under the regulation of different lengths of the C/EBP-dependent myelomonocytic growth factor (MGF) promoter (45). The following constructs were employed; pXP1, a promoterless construct; pMGF40, a 40 bp portion of the MGF promoter that lacks C/EBP sites; and pMGF65 and pMGF82 which contain one and two C/EBP binding sites, respectively. Two C/EBP binding sites are necessary for maximal C/EBP transactivation of the MGF promoter (45). As shown in Figure 1, primary CD-1 mouse keratinocytes transfected with pXP1 or pMGF40 demonstrated low luciferase activity in low calcium medium with a minimal 2 fold or less increase in luciferase expression in high calcium media. In contrast, keratinocytes transfected with pMGF65 or pMGF82 exhibited a 4- and 7-fold induction, respectively, of luciferase activity when switched from low to high calcium medium. In low calcium medium pMGF82 exhibited approximately 2-fold greater luciferase activity

than pMGF40 while in high calcium medium pMGF82 exhibited 8 fold greater activity than pMGF40. Thus, the increase in luciferase activity is dependent upon C/EBP binding sites in the MGF promoter indicating that the endogenous trans-activating activity of C/EBP is increased in primary mouse keratinocytes in high calcium medium.

C/EBPβ inhibits the growth and alters the cell morphology of BALB/MK2 keratinocytes

To determine whether C/EBPβ can influence keratinocyte growth, we examined the effect of the forced expression of C/EBPβ on BALB/MK-2 keratinocytes when cultured under proliferative conditions (low calcium medium). BALB/MK-2 keratinocytes were employed, as mouse primary keratinocytes cannot not be passaged in serum containing medium and they require high cell densities for growth. BALB/MK-2 keratinocytes are a nontransformed immortalized cell line that retains responsiveness to the modulation of growth arrest and terminal differentiation induced by increased calcium concentrations (53). An expression vector, pcDNA3-C/EBPβ, was constructed which placed the C/EBPβ cDNA under the regulation of the cytomegalovirus (CMV) promoter. The pcDNA3 vector also contains a neomycin resistance gene under the regulation of the SV40 promoter. Empty pcDNA3 or pcDNA3-C/EBPβ constructs were transfected into BALB/MK-2 keratinocytes and some plates from both groups were immunostained with a C/EBPβ specific antibody. At 24 and 48 hrs post-transfection, keratinocytes transfected with pcDNA3-C/EBPβ demonstrated a 5- and 9-fold increase in the number of cells staining positive for C/EBPβ when compared to cells transfected with empty pcDNA3, confirming that C/EBPβ was expressed from the construct. At 24 hrs after transfection, the cells were cultured in low calcium medium in the presence of G418 and, as shown in Figure 2A, after 3 days of G418 selection there were 75% fewer colonies/dish in cultures

transfected with pcDNA3-C/EBPβ compared to cultures transfected with empty pcDNA3. The number of colonies/dish continued to decrease and by 10 days the cultures transfected with pcDNA3-C/EBPβ demonstrated approximately 90% fewer colonies/dish than cultures transfected with empty pcDNA3. In addition to the decrease in the number of colonies/dish, the number of cells/colony in the cultures transfected with pcDNA3-C/EBPβ was also decreased. As shown in Figure 2B, there were 70-80% fewer cells/colony in the pcDNA3-C/EBPβ transfected cultures compared to the cultures transfected with the pcDNA3 vector control after 5, 7 and 10 days of G418 selection. As shown in Figures 3A and 3B, cells transfected with pcDNA3-C/EBPβ exhibited an enlarged and flattened morphology similar to the cell morphology observed in BALB/MK-2 cells switched to high calcium media. These results indicate that forced expression of C/EBPβ inhibits the growth and alters the cell morphology of BALB/MK2 keratinocytes. The above experiments were also conducted with C/EBPα and as shown in Figure 2A and 2B, C/EBPα also reduced the number of colonies/dish and cells/colony and these cells also demonstrated a enlarged flattened morphology. These results indicate that there is functional overlap with regard to the ability of C/EBPα and C/EBPβ to inhibit keratinocyte growth and induce alterations in cell morphology.

After 10 days of G418 selection some colonies in the plates transfected with pcDNA3-C/EBPβ survived and continued to proliferate. At least six colonies from each group were isolated and expanded in the presence of G418. Western analysis of whole cell lysates prepared from these pcDNA3-C/EBPβ colonies showed no increase in C/EBPβ protein expression when compared that from pcDNA3 colonies (data not shown). These results suggest that the surviving and proliferating G418 resistant

colonies originating from cultures transfected with pcDNA3-C/EBPβ have retained the neomycin resistance gene but have lost the ability to express C/EBPβ from the pcDNA3-C/EBPβ construct.

To provide additional evidence that C/EBPβ has the capacity to inhibit growth independent of the presence of G418 and long term G418 selection, we conducted BrdU labeling studies. Twenty four hours following transfection of BALB/MK2 keratinocytes with C/EBPβ, or empty vector control, transfected keratinocytes were selected for 24 hrs with G418 and the number of S-phase BrdU positive cells were determined at 0, 24 and 48 hrs post G418 removal. As shown in Figure 4, C/EBPβ transfected keratinocytes were growth inhibited and did not display any increase in the number of S-phase cells in the absence of G418 while empty vector transfected cells displayed a 4-5 fold increase in the number of BrdU S-phase positive cells. Kerainocytes that remained in the presence of G148 for 48 hours continued to be growth inhibited and displayed BrdU S-phase labeling indices similar to that observed at 0 hours after G418 removal. These results indicate that C/EBPβ induces growth inhibition independent of presence of G418 and long-term G418 selection.

C/EBPβ induces K1 and K10 expression in BALB/MK2 keratinocytes.

BALB/MK2 keratinocytes were transfected with empty pcDNA3 or pcDNA3-C/EBPβ to determine if C/EBPβ can alter the expression of differentiation-specific genes. We examined the expression of keratin 1 (K1) and keratin 10 (K10), two early markers of keratinocyte differentiation, and involucrin and loricrin, two markers which are expressed later in the differentiation program. Forty eight hours after transfection keratinocytes were immunostained for C/EBPβ, K1, K10, involucrin and loricrin. As shown in Figure 5 there was 9-fold increase in the number of C/EBPβ positive cells in

the keratinocytes transfected with pcDNA3- C/EBPβ vector compared to the keratinocyte cultures transfected with the empty pcDNA3. In addition there was a 5- and 3- fold increase in the number of K1 and K10 positive cells, respectively, in the keratinocytes transfected with pcDNA3-C/EBPβ. The number of cells staining positive for involucrin and loricrin were only minimally increased in the keratinocytes transfected with pcDNA3-C/EBPβ. Similar results were obtained when the cells were immunostained 72 hrs after transfection (data not shown). The cells that stained positive for C/EBPβ, K1 and K10 were detected as isolated single cells despite the fact that the BALB/MK2 keratinocytes were transfected when the cells were 30% confluent and immunostained 48 hours later when they were 100% confluent. These data indicate that increased expression of C/EBPβ is associated with increases in the expression of K1 and K10 proteins and further supports our notion that C/EBPβ modulates keratinocyte growth and the early events in differentiation.

To assure that K1 expression occurs in the C/EBPβ transfected cell populations double immunofluorescence detection studies were conducted. As shown in Figure 6A, C/EBPβ transfected positive cells demonstrated bright green fluorescence nuclear staining and these same cells co-expressed K1 as indicated by intense red fluorescence cytoplasmic staining (Figure 6B). Double immunofluorescence staining showed that 26% of the C/EBPβ positive cells co-expressed K1 and this result is similar to the value reported in Figure 5. It was observed that the brightest C/EBPβ transfected cells generally did not display the strongest K1 signal but rather the medium to lower intensity C/EBPβ transfected cells produced the greatest K1 signal, suggesting very high levels of C/EBPβ may be inhibitory to K1 expression. Empty vector transfected cells demonstrated very few C/EBPβ or K1 positive cells.

C/EBPβ deficient mice demonstrate abnormalities in keratinocyte proliferation and differentiation.

To gain further insight into the functional role of C/EBPβ in epidermal keratinocytes, we analyzed the epidermis of mice which carry a targeted deletion of C/EBPβ. Since both C/EBPα and C/EBPβ are expressed in mouse epidermis, it was of interest to first determine whether the absence of the C/EBPβ protein influenced the level of expression of the C/EBPα protein. Whole cell epidermal lysates were prepared from three C/EBPβ null, three heterozygous and three wildtype mice. Representative Western blot analyses are shown in Figure 7A and 7B. As shown in Figure 7A, C/EBPα protein levels (42 kDa) were similar in all three genotypes. The 30 kDa C/EBPα truncated protein level appears to be decreased in C/EBPβ null mice, however other western analysis did not demonstrate such a decrease suggesting that the observed decrease may be an artifact due to poor transfer or poor wetting of the membrane with the chemiluminescence solutions. As expected, C/EBPβ (36 kDa and 21 kDa) proteins could not be detected in epidermal lysates isolated from C/EBPβ null mice while their levels in the C/EBPβ heterozygous mice were intermediate between the C/EBPβ deficient and wildtype mice (Fig 7B). Thus, the absence of the C/EBPβ protein in the epidermis has little or no effect on epidermal C/EBPα protein levels.

As shown in Table 1, C/EBPβ deficient mice demonstrated a mild epidermal hyperplasia. There were statistically significant ($p < 0.05$) increases in epidermal thickness, the number of nucleated cell layers, as well as the number of BrdU S-phase positive keratinocytes in the interfollicular epidermis of C/EBPβ deficient mice compared with wildtype mice. To determine if the observed abnormalities in

keratinocyte proliferation are accompanied by alterations in keratinocyte differentiation, the epidermis was isolated from the wildtype and C/EBPβ deficient mice and western analysis was conducted to determine whether the expression of K5, K1, K10, the cornified envelope proteins loricrin and involucrin were altered. We chose to examine K5 as it is expressed in the basal layer, while K1 and K10 are expressed upon transition from the basal to the spinous layer of the epidermis. Involucrin and loricrin are expressed later in the differentiation program in the granular layers of the epidermis. As shown in Figure 8A and 8B, there were modest but consistent decreases in K1 and K10 levels (45% and 35% respectively, $p<0.05$) in the epidermis of C/EBPβ deficient mice compared to wildtype mice as determined by laser densitometric analysis. In contrast, the levels of K5, loricrin and involucrin in epidermal preparations isolated from C/EBPβ deficient were similar to wildtype mice ($p>0.05$). These results indicate that C/EBPβ deficient mice display abnormalities in keratinocyte growth, K1 and K10 expression in the interfollicular epidermis, and these abnormalities occur in the absence of alterations in the level of C/EBPα.

Primary keratinocytes isolated from C/EBPβ deficient mice display decreases in K1 and K10 expression

In low calcium medium, attached keratinocytes resemble the basal keratinocytes of the epidermis. The attached keratinocytes are a proliferative population and when an attached keratinocyte terminally differentiates, it spontaneously detaches from the plate and is replaced by the attached proliferative keratinocytes. Therefore, two distinct populations of keratinocytes, the spontaneously detached terminally differentiated cells and the attached proliferative undifferentiated cells can be evaluated. Keratinocytes from wildtype and C/EBPβ deficient newborn mice were isolated and the ability of

these primary keratinocytes to undergo growth arrest and differentiation was examined. All experiments used pooled keratinocytes of a single genotype and were repeated at least 3 times. In low calcium medium C/EBPβ deficient keratinocytes grew to 50% higher saturation density than wildtype keratinocytes and at confluence C/EBPβ deficient keratinocytes were smaller and more polygonal in shape with more highly distinct intercellular spaces. Confluent cultures of C/EBPβ deficient keratinocytes exhibited a 45-50% decrease in DNA synthesis as determined by ^{3}H-thymidine incorporation into DNA and a concomitant 30% decrease in the number of spontaneously detached differentiated cells when compared to the wildtype keratinocytes. Based on the decreased number of spontaneously detached cells and the increased number of attached cells at confluence, we speculated that C/EBPβ deficient keratinocytes may have an attenuated ability to initiate or execute early events in the process of keratinocyte differentiation.

To characterize defects in differentiation at the molecular level, C/EBPβ deficient and wildtype primary keratinocytes were cultured in low calcium medium for six days and then the spontaneously detached and attached proliferative populations of cells were collected and lysates were prepared for western analysis. In addition, we induced differentiation in attached keratinocytes by placing these cells in suspension culture for 16 hours and collected the cells for western analysis on day six (12). A comparison of the expression of K1, K10, involucrin and loricrin in C/EBPβ deficient keratinocytes versus wildtype keratinocytes revealed striking differences in the expression of K1 and K10 (Figure 9A). The spontaneously detached, attached and suspension cultured C/EBPβ deficient keratinocytes expressed 40%, 70%, and 95% less K1 than the wildtype counterparts. Likewise, K10 expression was dramatically

decreased. Compared to their wildtype keratinocyte counterparts, K10 expression was decreased by 50, 30 and 95% in spontaneously detached, attached and suspension cultured C/EBPβ deficient keratinocytes. Overexposure of the K1 and K10 signals in the detached keratinocytes was necessary to produce detectable signals for K1 and K10 in attached and suspension cultured cells. Densitometric analysis of films produced from shorter exposures revealed that the decreases in K1 and K10 in detached C/EBPβ deficient keratinocytes versus the detached wildtype keratinocytes were 70% and 80%, respectively (data not shown). As shown in Figure 9A, involucrin and loricrin protein expression was similar in the C/EBPβ deficient keratinocytes when compared to the wildtype counterpart, indicating that the lack of C/EBPβ does not cause a general decrease in all markers of differentiation. Based on these results, the early differentiation specific events involving K1 and K10 expression that occur upon transition from the basal to the spinous layer of the epidermis are preferentially altered by the deletion of the C/EBPβ gene. Since keratin expression is predominately regulated at the level of transcription (17, 38), we conducted northern blot analysis for K1 mRNA on RNA isolated from the attached and suspension cultured keratinocytes. As shown in Figure 9B and 9C, C/EBPβ deficient keratinocytes display significantly decreased K1 mRNA levels compared to the wildtype keratinocytes. These results were consistently observed in experiments with different preparations of isolated primary newborn keratinocytes. These results support a role for C/EBPβ in the regulation of K1 mRNA levels.

Primary keratinocytes isolated from C/EBPβ deficient mice are resistant to calcium-induced growth arrest

Since C/EBPβ appeared to influence the early events in keratinocyte differentiation and

C/EBPβ deficient mice displayed an epidermal hyperplasia, we examined whether C/EBPβ deficient keratinocytes displayed defects in their ability to undergo calcium-induced growth arrest. Growth arrest of primary keratinocytes is an early event in the process of keratinocyte differentiation, occurring prior to the expression of early markers of differentiation, such as K1 and K10. Keratinocytes isolated from the epidermis of wildtype and C/EBPβ deficient mice were shifted from medium containing 0.05 mM calcium to media containing 0.12 mM calcium. The cells were harvested at 6, 12 and 24 hours and growth arrest was monitored by one-hour pulse labeling with ^{3}H-thymidine during the last hour prior to harvest. As shown in Figure 10, when wildtype keratinocytes were switched to medium containing 0.12 mM calcium, growth arrest occurred very rapidly; by 6 hours there was a 35% decrease in DNA synthesis and by 12 hrs DNA synthesis was decreased by greater than 80%. In contrast, C/EBPβ deficient keratinocytes were resistant to calcium induced growth arrest. As shown in Figure 10, there was no decrease in DNA synthesis in C/EBPβ deficient keratinocytes at 6 hrs after the switch to medium containing 0.12 mM calcium and only a 35% decrease at 12 hours. However, by 24 hours DNA synthesis was decreased to a level similar to that observed in the wildtype keratinocytes. C/EBPβ deficient keratinocytes shifted to medium containing 2.0 mM calcium demonstrated a similar resistance to the growth arrest effects of calcium (data not shown). To determine if C/EBPα underwent a compensatory upregulation in the C/EBPβ deficient keratinocytes, western blot analysis was conducted on cell extracts isolated from wildtype and C/EBPβ deficient keratinocytes at 0, 6, 12 and 24 hrs post high calcium shift. No differences in C/EBPα levels were observed between wildtype and C/EBPβ deficient keratincytes (data not shown). Recent evidence indicates the $p21^{Cip1/WAF1}$ plays an

important role in regulating both keratinocyte growth and differentiation (9). Following the addition of high calcium to the media, it has been shown that $p21^{Cip1/WAF1}$ is rapidly induced and produces a block in cell cycle progression at the G1 phase. However, keratinocyte differentiation is also blocked by $p21^{Cip1/WAF1}$ and does not ensue until $p21^{Cip1/WAF1}$ levels return to basal levels. Thus it has been proposed that $p21^{Cip1/WAF1}$ couples growth arrest and differentiation in keratinocytes (9). Western analysis of whole cell lysates from wildtype and C/EBPβ deficient keratinocytes for $p21^{Cip1/WAF1}$ protein levels revealed that $p21^{Cip1/WAF1}$ levels increased 2-3 fold at 6 hrs after the switch to 0.12 mM calcium in both groups and subsequently decreased within 24 hrs to levels lower than that observed before the calcium switch (data not shown) indicating the $p21^{Cip1/WAF1}$ expression is not altered in the C/EBPβ deficient keratinocytes.

DISCUSSION

Within the mouse epidermis, C/EBPβ is exclusively detected in the nuclei of suprabasal keratinocytes (31). This highly compartmentalized location of C/EBPβ suggested that C/EBPβ plays a role in the regulation of genes involved in or specifically expressed during the process of squamous differentiation (31). Our current results provide the first evidence that C/EBPβ can directly modulate the program of squamous differentiation in the epidermis and in isolated keratinocytes. We propose that C/EBPβ is involved in the regulation of the early stages of squamous differentiation of epidermal keratinocytes based on the following experimental evidence; i) forced expression of C/EBPβ inhibits growth and induces K1 and K10 in BALB/MK2 keratinocytes and has minimal effects on later stage differentiation markers, ii) differentiated C/EBPβ deficient primary keratinocytes, both spontaneous detached and suspension culture-induced, demonstrate striking decreases in K1 and K10 expression with minimal alterations in later stage differentiation markers, iii) C/EBPβ deficient primary keratinocytes display resistance to calcium-induced growth arrest, and iv) direct analysis of C/EBPβ deficient mouse skin revealed a hyperplastic epidermis and decreases in K1 and K10 expression with minimal differences in involucrin, loricrin or K5 expression. Thus, results derived from both mutating and overexpressing C/EBPβ support a functional role for the protein in the regulation of growth arrest and K1 and K10 expression in keratinocytes.

While our findings identify a functional role for C/EBPβ in the regulation of K1 and K10 levels, it is not known if this is a direct effect of C/EBPβ within the promoter regions of K1 and K10 or if C/EBPβ is indirectly modulating K1 an K10 levels. However, the levels of both K1 and K10 are largely regulated at the level of

transcription (17, 38). Sequence analysis of the K1 and K10 promoters (22, 35) revealed that both promoters contain several potential C/EBP binding sites. Utilizing the C/EBP dependent MGF promoter, we demonstrated that the transactivation activity of endogenous C/EBP in keratinocytes increases under conditions known to induce growth arrest and differentiation. In addition, K1 mRNA levels are dramatically decreased in C/EBPβ deficient keratinocytes. Taken together, these findings suggest that C/EBPβ may directly modulate the transcription of K1 and K10. Regardless, it is clear that C/EBPβ influences K1 and K10 levels and that additional factors also contribute to the regulation of K1 and K10 as their expression was not completely abolished in C/EBPβ-deficient epidermis or primary keratinocytes. Skn-1a, a member of the POU domain family of transcription factors, is expressed in the suprabasal layers of the epidermis and has been shown to activate the K10 promoter in HeLa cells (2), however, keratinocytes from Skn-1a deficient mice do not demonstrate alterations in K10 levels (3). With regard to K1, an AP-1/steroid site has been identified in the 3' flanking region of the K1 gene and this element imparts some responsiveness to calcium induced differentiation (21, 24, 40). c-Fos, a component of AP-1 that has been proposed to function in the terminal stages of epidermal differentiation, also exhibits exclusive expression in the three cells of the EPU (16). Fos and C/EBP can form an association *in vitro* (20) which could impart another level of complexity to the regulation of K1. Further work will be required to determine whether bona fide C/EBP binding sites exist in the K1 and K10 promoters and if C/EBP interacts with other transcription factors.

The alterations in K1 and K10 expression observed in isolated C/EBPβ deficient primary keratinocytes were more striking than the more modest changes

observed in the epidermis of C/EBPβ deficient mice. It is possible that the disruption of epidermal homeostatic mechanisms that tend to attenuate the expression of genetic defects in intact skin may allow for a more full expression of the defect in keratinocytes in primary culture. Phenotypic differences in intact skin versus primary keratinocytes have been reported for other null mice (27).

Growth arrest of primary keratinocytes is an early event in the process of keratinocyte differentiation occurring prior to the expression of early markers of differentiation, such as K1 and K10. Our results indicate that C/EBPβ deficient keratinocytes exhibit growth abnormalities in intact skin as well as in primary culture. In addition, we have found the forced expression of C/EBPα also inhibits keratinocyte growth. Since C/EBPα is expressed in the basal keratinocytes of the epidermis, it may initiate growth inhibition and then C/EBPβ maintains growth arrest and also induces the expression of K1 and K10 upon upward movement of the basal keratinocyte to the suprabasal layers of the epidermis. Since both C/EBPα and C/EBPβ are expressed in suprabasal keratinocytes, it is possible that they form heterodimers and cooperate to induce growth arrest. Further studies are necessary to determine whether C/EBPα and C/EBPβ induce growth arrest through different mechanisms. $p21^{Cip1/WAF1}$ is considered to be a factor in keratinocyte growth arrest and differentiation (9). In colorectal cancer cells $p21^{Cip1/WAF1}$ is induced via a pathway involving C/EBPβ (8) While we did observe the normal characteristic increase in $p21^{Cip1/WAF1}$ levels followed by a decrease to below control levels in keratinocytes in high calcium medium, we did not observe any major differences between wildtype and C/EBPβ deficient mice. These results suggest that the deletion of the C/EBPβ gene does not interfere with the expression of $p21^{Cip1/WAF1}$ in primary keratinocytes and provide evidence that

keratinocyte growth can be regulated by multiple molecular mechanisms. The retinoblastoma (Rb) family of proteins are important regulators of the cell cycle and recently Rb has been shown to influence adipocyte differentiation through its physical interaction with C/EBP (7). Rb family members can influence epidermal differentiation and keratinocyte proliferation (32) and as such may represent potential target proteins for C/EBPβ interactions and growth inhibition. A recent paper by Paramio *et al* (33) demonstrated that K10 expression but not K12, K14 and K16 expression, inhibits keratinocyte proliferation through an Rb pathway. These authors suggest that the complex differential expression of cytokeratins that occurs during squamous differentiation may be important in cell cycle regulation. The altered regulation of K10 in C/EBPβ deficient keratinocytes may contribute to the altered growth characteristics of these cells. Recently, it has been demonstrated that C/EBPα can interact with with Rb family member p107 and this interaction results in the disruption of E2F/p107 S-phase complexes (51). The disruption of these complexes is associated with C/EBPα induced growth arrest in hepatocytes of newborn mice. Whether C/EBPα and C/EBPβ can interact with p107 and alter cell cycle progression in keratinocytes is an area of future study. While further studies are required to discern the downstream pathway through which C/EBPβ regulates K1, K10 and growth arrest, our study provides novel fundamental insights into the function of C/EBPβ in the early events of squamous differentiation.

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Table 1. Altered epidermal keratinocyte proliferation in C/EBPβ deficient mice

Genotype	Epidermal Thickness (um)	Nucleated Cell layers	Labeling index (% S-phase)
C/EBPβ +/+	13.1 ± 0.7	2.0 ± 0.2	4.2 ± 1.5
C/EBPβ -/-	21.3 ± 2.7**	3.0 ± 0.4**	7.6 ± 2.8*

Each value represents the mean ± standard deviation from 5 mice/group.

* value is significantly different from corresponding wildtype value $p<0.05$,

* * value is significantly different from corresponding wildtype value $p<0.001$ as determined by two tailed Student's t test.

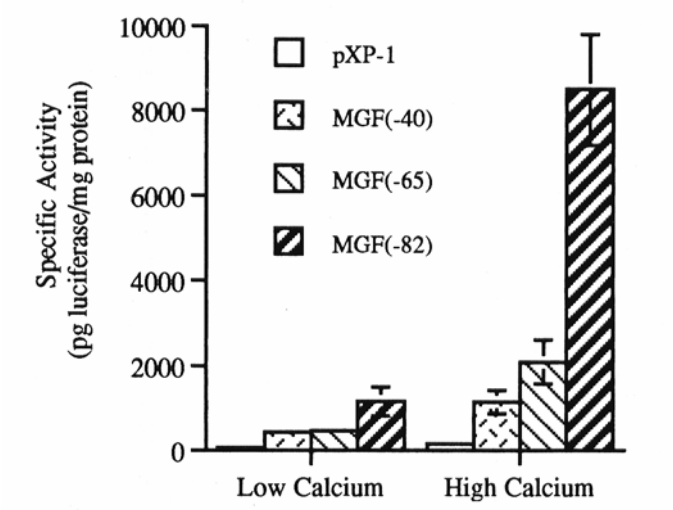

Fig. 1 *Transactivation potential of endogenous C/EBP proteins in primary keratinocytes.* Three days after plating primary CD-1 keratinocytes were transfected with the indicated promoter/luciferase reporter constructs. Keratinocyte cultures were shifted to high calcium medium or maintained in low calcium medium for 48 hours. Cells were then harvested and the luciferase activity was determined. Results are expressed as the mean ± standard deviation of a representative experiment with 3 plates/group.

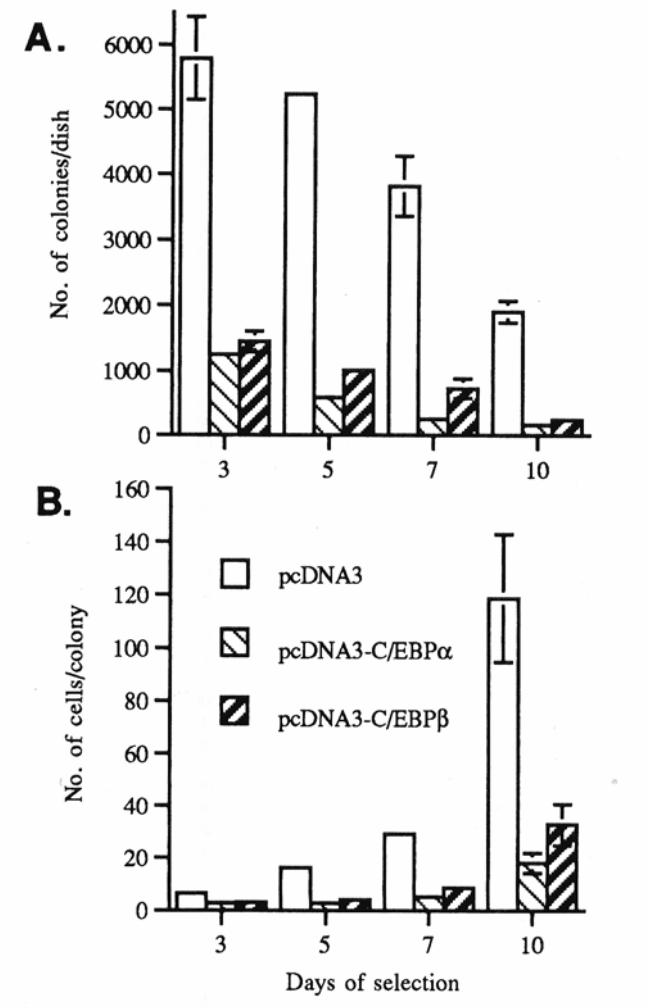

Fig. 2 *Forced expression of C/EBPβ and C/EBPα inhibits BALB/MK2 keratinocyte growth.*

BALB/MK2 keratinocytes were transfected with empty pcDNA3, pcDNA3-C/EBPβ, or pcDNA3-C/EBPα, subsequently sub-cultured in low calcium media in the presence of 500 μg/ml G418. The number of colonies per dish and the number of cells per colony were determined at day 3, 5, and 7 and 10 of G418 selection. Data are expressed as mean ± standard deviation of a representative experiment done in triplicate for each group. A.) Number of colonies/dish. B.) Number of cells/colony.

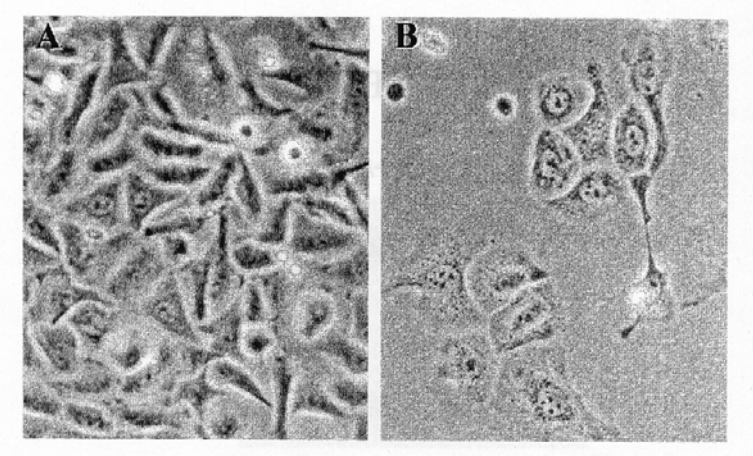

Fig. 3 *Forced expression of C/EBPβ alters BALB/MK2 keratinocyte morphology.* BALB/MK2 keratinocytes were transfected with empty pcDNA3 or pcDNA3-C/ EBPβ and subsequently sub-cultured in low calcium media in the presence of 500 μg/ml G418. At day 7 of G418 selection photographs of colonies (200X) were taken of: A.) BALB/MK2 keratinocytes transfected with empty pcDNA3, and B.) BALB/MK2 keratinocytes transfected with pcDNA3-C/EBPβ.

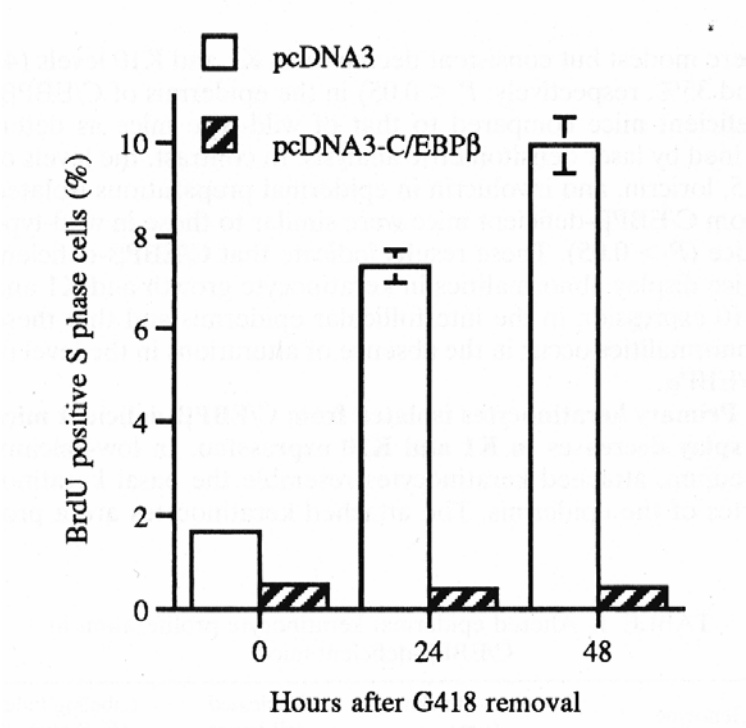

Fig. 4 *Transient transfection of BALB/MK2 keratinocytes with pcDNA3-C/EBPβ inhibits growth as indicated by the number of BrdU S-phase cells.*

Twenty four hours following transfection of BALB/MK2 keratinocytes with C/EBPβ, or empty vector control, transfected keratinocytes were selected for 24 hrs with G418 and the number of S-phase BrdU positive cells were determined at 0, 24 and 48 hrs post G418 removal. The number of BrdU S-phase positive cells was quantitated/1000 cells and the results are expressed as the number of BrdU S-phase positive cells/1000 cells x 100.

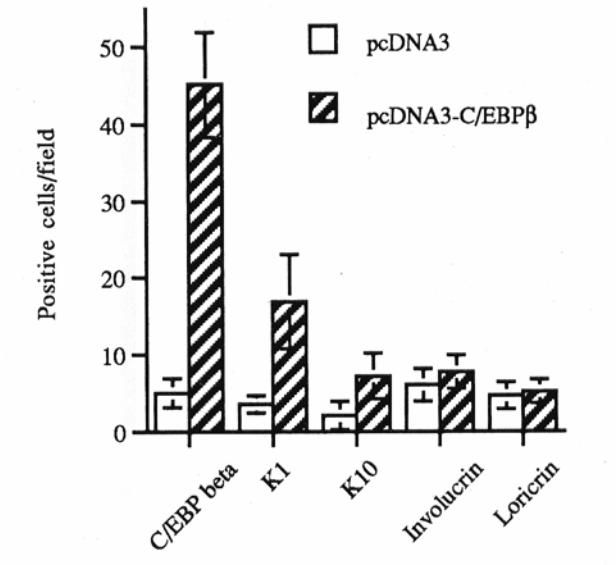

Fig. 5 *Transient transfection of BALB/MK2 keratinocytes with pcDNA3-C/EBPβ increases K1 and K10 expression.*

BALB/MK2 keratinocytes were cultured in low calcium medium and transfected with empty pcDNA3 or pcDNA3-C/EBPβ. Immunochemical staining for C/EBPβ, involucrin, loricrin, keratin 1 and keratin 10 was conducted at 48 hours after transfection. Single dark-brown-stained positive cells were quantitated in 10 fields (100X magnification) per dish. Data were expressed as positive cells/field (mean ± standard deviation). The number of C/EBPβ, K1, and K10 positive cells in the pcDNA3-C/EBPβ transfected cultures was statistically significantly different than that of pcDNA3 transfected cultures ($p<0.01$, two tailed Student's t-test).

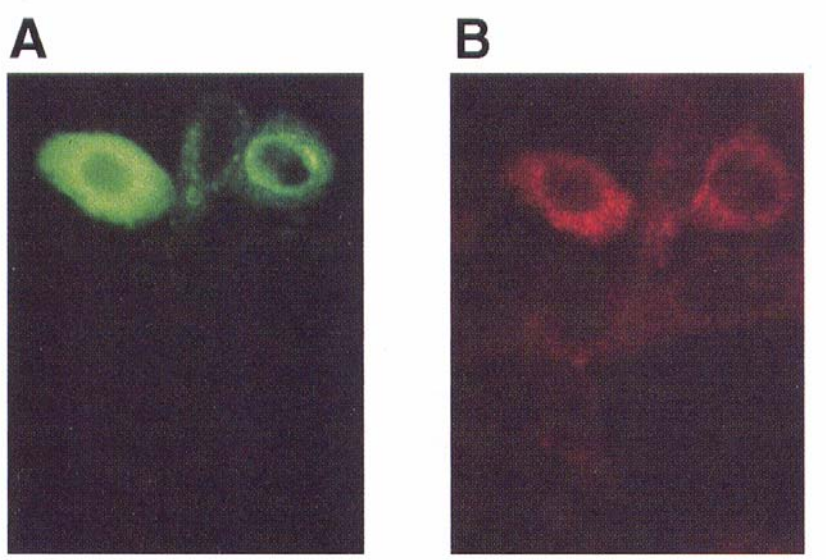

Fig. 6 *Immunofluorescence detection of the co-expression of C/EBPβ and K1 in pcDNA3-C/EBPβ transfected BALB/MK2 keratinocytes.*

BALB/MK2 keratinocytes were transiently transfected with pcDNA3-C/EBPβ and processed for detection of C/EBPβ and K1 co-expression as described in the Methods section. A) C/EBPβ staining (FITC). B) K1 staining (Texas Red)

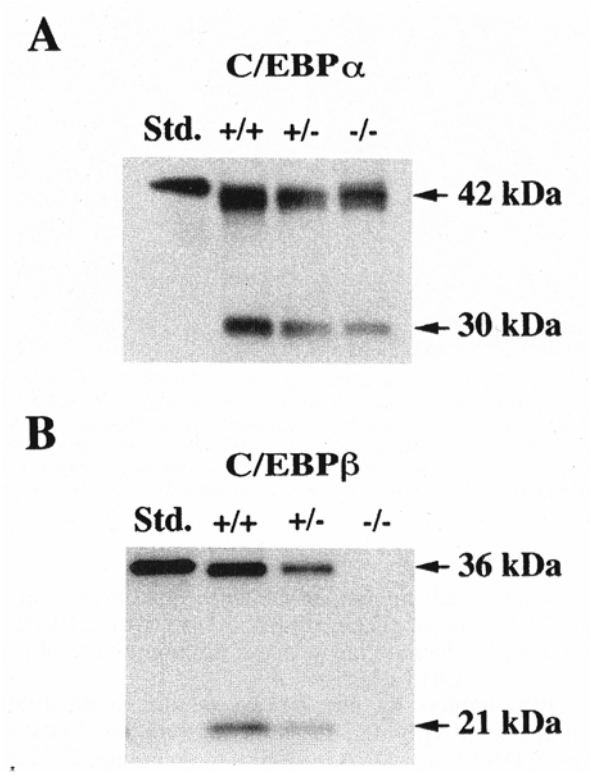

Fig. 7 *C/EBPα and C/EBPβ expression in the epidermis of C/EBPβ wildtype, heterozygous and deficient adult mice.*

Whole cell epidermal lysates were prepared from the epidermis of adult female mice and Western analysis conducted. A.) C/EBPα protein in wildtype (+/+), C/EBPβ heterozygous (+/-) and C/EBPβ deficient (-/-) mice. B.) C/EBPβ protein in wildtype (+/+), C/EBPβ heterozygous (+/-) and C/EBPβ deficient (-/-) mice. C/EBPα and C/EBPβ standards are histidine-tagged and migrate more slowly than the native protein.

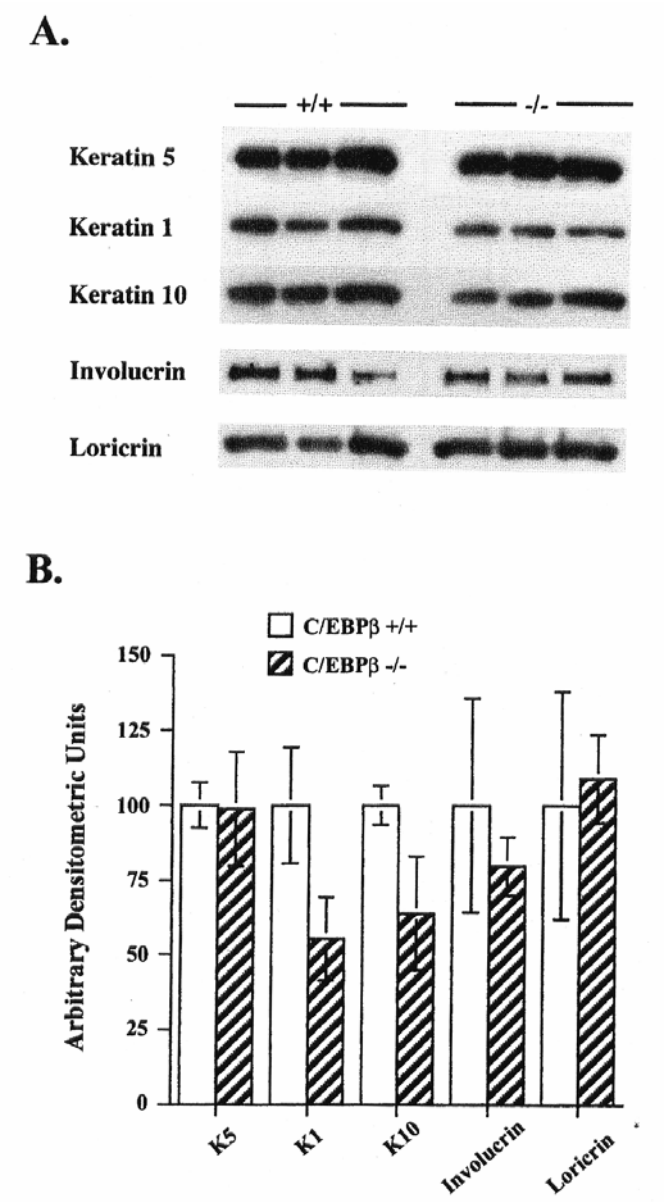

Fig. 8 *C/EBPβ deficient mice demonstrate alterations in epidermal K1 and K10 expression.*

Whole cell epidermal lysates were prepared from the epidermis of adult wildtype and C/EBPβ deficient female mice and A) Western analysis conducted with specific antisera as indicated. Epidermal lysates from wildtype and C/EBPβ deficient mice are represented by +/+ and -/-, respectively and each lane contains protein from a different mouse.

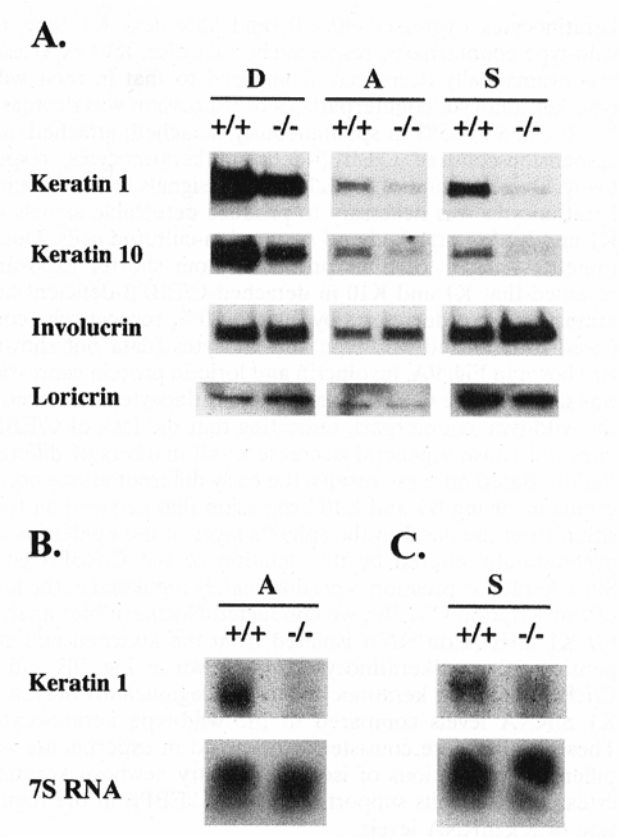

Fig. 9 *Altered expression of K1 and K10 in attached, spontaneously detached and suspension-cultured primary keratinocytes from C/EBPβ deficient mice.*

A) Primary newborn keratinocytes were maintained in low calcium medium. At day 6 after plating, attached cells (A), spontaneously detached cells (D), and 16 hr suspension cultured cells (S) (see text) were collected, whole cell lysates prepared, and Western analysis conducted as indicated. Lysates from wildtype and C/EBPβ deficient samples are represented by +/+ and -/-, respectively. Primary newborn keratinocytes were maintained in low calcium medium. At day 7 after plating, (B) attached cells , and (C) 16 hr suspension cultured cells (see text) were collected, RNA isolated and northern blot analysis for K1 conducted.

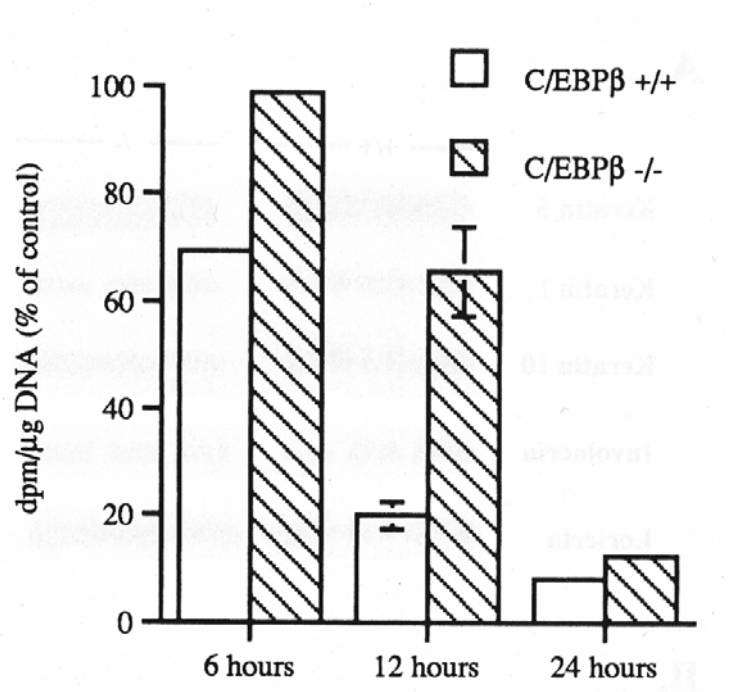

Fig. 10 *C/EBPβ deficient epidermal keratinocytes are resistant to calcium-induced growth arrest in vitro.*

Primary keratinocytes were cultured in low calcium medium for 6-7 days and then either switched to high calcium medium or re-fed with low calcium medium. Cultures were pulse labeled with [^{3}H-methyl]-thymidine for 1 hour prior to harvest. Dpm/µg DNA was determined from triplicate plates/group, and the data is presented as percentage of control [^{3}H-methyl]-thymidine incorporation in keratinocytes cultured in low calcium medium at each time point.