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ARTICLES

Expression and Function of CCAAT/Enhancer Binding Proteinβ (C/EBPβ) LAP and LIP Isoforms in Mouse Mammary Gland, Tumors and Cultured Mammary Epithelial Cells

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Abstract CCAAT/Enhancer binding proteins (C/EBPs) play important roles in the regulation of cell growth and differentiation. This study investigated the expression and function of C/EBPβ isoforms in the mouse mammary gland, mammary tumors, and a nontransformed mouse mammary epithelial cell line (HC11). C/EBPβ mRNA levels are 2–5-fold higher in mouse mammary tumors derived from MMTV/c-neu transgenic mice compared with lactating and involuting mouse mammary gland. The ''full-length'' 38 kd C/EBPβ LAP (''Liver-enriched Activator Protein'') isoform is the predominant C/EBPβ protein isoform in mammary tumor whole cell lysates, however, the truncated 20 kd C/EBPβ LIP (''Liver-enriched Inhibitory Protein'') isoform is also present at detectable levels (mean LAP:LIP ratio 5.3:1). The mammary tumor C/EBPβ LAP:LIP ratio decreases 70% (from 5.3:1 to 1.6:1) when lysate preparation is switched from a rapid whole cell lysis protocol to a multistep nuclear/cytoplasmic fractionation protocol. In contrast to mammary tumors, only the C/EBPβ LAP isoform is detectable in the mammary gland whole cell and nuclear lysates; the truncated ''LIP'' isoform is undetectable regardless of isolation protocol. Ectopic over expression of C/EBPβ LIP or C/EBPβ LAP did not alter HC11 growth rates. However, C/EBPβ LIP over expressing HC11 cells (LAP:LIP ratio of ∼1:1) exhibited a consistent 2–4 h delay in G_0/S phase transition. C/EBPβ LIP overexpressing HC11 cells did not express β-casein mRNA (mammary epithelial cell differentiation marker) in response to lactogenic hormones. This defect in β-casein expression was not corrected by carrying out the differentiation protocol in the presence of an artificial extracellular matrix. These results demonstrate that the ''full-length'' C/EBPβ LAP isoform is the predominant C/EBPβ protein isoform expressed in mouse mammary gland in vivo and mouse mammary epithelial cell cultures in vitro. C/EBPβ LIP detected in mammary tumor lysates may result from in vivo production or ex vivo isolation-induced proteolysis of C/EBPβ LAP. Ectopic overexpression of C/EBPβ LIP (LAP:LIP ratio of ∼1:1) inhibits mammary epithelial cell differentiation (β-casein expression). J. Cell. Biochem. 82: 357–370, 2001.

Key words: CCAAT/Enhancer Binding Proteins; C/EBP; LAP; LIP; mammary epithelial cells

CCAAT/Enhancer Binding Proteins (C/EBPs) are a widely expressed, highly conserved family of leucine zipper type (bZIP) DNA binding proteins [Hurst, 1994; Lekstrom-Himes et al., 1998]. Six family members have been characterized including C/EBPα, C/EBPβ (also called CRP2, NF-IL6, LAP, AGP/EBP, IL6BP, or NF-M), C/EBPδ (also called CRP3, NF-IL6b, or CELF), C/EBPε, C/EBPγ, and C/EBP-Homologous Protein10 (CHOP10, GADD153) [Lekstrom-Himes et al., 1998]. Most C/EBPs are encoded by intronless genes and exhibit significant homology in the basic and leucine zipper regions [Hurst, 1994; Lekstrom-Himes et al., 1998]. C/EBPs bind to DNA as homodimers or as heterodimers with other C/EBP family members or other bZIP proteins such as c-fos and CREB/ATF [Williams et al., 1991; Hurst, 1994; Lekstrom-Himes et al., 1998]. Functional C/EBP binding sites have been identified in the promoters of genes that function in

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cell growth, such as c-fos [Sealy et al., 1997] and genes that are expressed in differentiated cells, such as phosphoenolpyruvate carboxykinase (PEPCK) [Croninger et al., 1998] and β-casein [Doppler et al., 1995].

In addition to a direct role in specific gene transcription, C/EBPs also influence cellular biochemical pathways through protein–protein interactions with key regulatory proteins [Chen et al., 1996a, 1996b]. For example, C/EBPβ (NFIL6) binds to the hypophosphorylated form of the Retinoblastoma protein (Rb) and this interaction plays a key role in facilitating the differentiation of 3T3-L1 preadipocytes [Chen et al., 1996a, 1996b]. C/EBPβ/Rb interaction has also been hypothesized in the control of mammary epithelial cell growth and differentiation [Zahnow et al., 1997]. In addition, C/EBPs may influence cell fate determination by directly binding to chromosomal structural elements. C/EBPα directly associates with C/EBP consensus binding sites within centromeres during mitosis preceding 3T3-L1 pre-adipocyte differentiation [Tang et al., 1999].

We previously investigated the expression of C/EBP family members in the normal mouse mammary gland and found that C/EBPβ expression was highly induced during gestation and postweaning involution [Gigliotti and DeWille, 1998; Gigliotti et al., 1999]. Other labs have also observed the induction of C/EBPβ gene expression in the mammary gland during gestation [Raught et al., 1995] and involution [Sabatakos et al., 1998]. Two independent studies have reported that mammary gland ductal morphogenesis, lobuloalveolar development, and functional differentiation are defective in female C/EBPβ knockout mice [Robinson et al., 1998; Seagroves et al., 1998]. Collectively these results demonstrate that C/EBPβ plays an essential role in mammary gland development and function.

The C/EBPβ gene is transcribed into a single 1.4 kb mRNA [Cao et al., 1991; Descombes and Schibler, 1991; O'Rourke et al., 1997; Gigliotti and DeWille, 1998; Gigliotti et al., 1999]. At the protein level, however, multiple C/EBPβ isoforms, varying in size from 14 to 38 kD, have been reported [Descombes and Schibler, 1991; Raught et al., 1995]. The C/EBPβ isoforms include two "full-length" LAP (Liver-enriched Activator Protein) isoforms (38 and 34 kD) and one truncated 21 kD LIP (Liver-enriched Inhibitory Protein) isoform [Descombes and Schibler, 1991]. In addition, a second truncated 14 kD C/EBP isoform has been identified in macrophages [Baer et al., 1998]. Both the p14 and p21 truncated C/EBPβ isoforms retain the carboxy terminal bZIP and DNA binding domains but lack the amino terminal transactivation domain. Several reports indicate that the ratio of C/EBPβ isoforms present in a cell can be altered by a variety of cellular conditions [Descombes and Schibler, 1991; Raught et al., 1995]. This is potentially significant as evidence indicates that the amino terminal truncated C/EBPβ isoforms function as dominant-negative inhibitors of the full-length C/EBPβ LAP isoform, even when present at substoichiometric levels (i.e., LAP:LIP ratio of 5:1) [Descombes and Schibler, 1991; Raught et al., 1995; Baer et al., 1998]. Although the cellular regulation of C/EBP LAP and LIP levels is controversial, the capacity of C/EBP LIP to alter C/EBP LAP function has been directly demonstrated in 3T3-L1 cells. C/EBPβ LAP plays an essential role in the early commitment phase of 3T3-L1 differentiation [Cao et al., 1991; Wu et al., 1995]. Ectopic over expression of C/EBPβ LIP, resulting in a C/EBPβ to LIP ratio approximating 1:1, blocks 3T3-L1 differentiation, presumably through inhibition of C/EBPβ LAP function [Yeh et al., 1995].

Since C/EBPβ LAP function is also essential for the development and function of the mammary gland [Robinson et al., 1998; Seagroves et al., 1998], the presence of C/EBPβ LIP could alter normal mammary epithelial cell differentiation and influence cell fate. Elevated C/EBPβ LIP has been reported in the mammary gland during gestational proliferation, in mouse mammary tumors, and in mouse mammary tumor-derived cell lines [Raught et al., 1995, 1996]. Elevated C/EBPβ LIP levels have also been reported in infiltrating human ductal carcinomas of the breast [Zahnow et al., 1997]. These reports suggest that the presence of C/EBPβ LIP in mammary cells is associated with increased proliferation and/or tumorigenesis. The potential physiological significance of C/EBPβ LIP has led to a number of studies investigating the cellular mechanisms underlying C/EBPβ LIP production. It was initially hypothesized that C/EBPβ LIP was produced by a poorly understood "leaky ribosome scanning" mechanism [Descombes and Schibler, 1991; Ossipow et al., 1993; Raught et al.,

1996]. This is plausible as the C/EBPβ mRNA transcript contains an internal AUG, which is contained within an optimal Kozak sequence [Descombes and Schibler, 1991; Ossipow et al., 1993; Raught et al., 1996]. If alternate translation initiation did occur at the internal AUG it would result in the production of a protein approximately the size of C/EBPβ LIP [Descombes and Schibler, 1991; Ossipow et al., 1993; Raught et al., 1996].

Recent reports have suggested an alternate explanation for C/EBPβ LIP production [Welm et al., 1999]. Lincoln et al. have reported that C/EBPβ (like C/EBPα) contains a highly conserved upstream open reading frame (uORF) that represses the translation of the full-length C/EBP downstream translation product [Lincoln et al., 1998]. Mutational alterations in the C/EBPβ LAP μORF and in the "full-length" C/EBPβ LAP AUG initiating codon did not induce alternate translation at the downstream AUG and production of the truncated C/EBPβ LIP translation product [Lincoln et al., 1998]. These results suggested that the production of the truncated C/EBPβ LIP translation product via an internal translation initiation mechanism was unlikely [Lincoln et al., 1998]. In a follow-up report Lincoln et al. next demonstrated that the full-length C/EBPβ LAP isoform can be quantitatively cleaved by endogenous proteases to produce truncated 20 and 14 kd C/EBPβ proteolytic products [Baer et al., 1998; Baer and Johnson, 2000]. Generation of the C/EBPβ proteolytic products from cell lysates was dependent, in large part, on the isolation method [Baer and Johnson, 2000]. These results suggest that some or all of the C/EBPβ LIP detected by Western blot is the result of isolation-induced proteolysis of C/EBPβ LAP.

The initial objective of this study was to investigate the expression of C/EBPs in general, and C/EBPβ LAP and LIP isoforms in particular, in the mouse mammary gland and mammary tumors in vivo. The results indicate that C/EBPβ LAP is the major C/EBP family member expressed in mammary gland and mammary tumors. The truncated dominant-negative C/EBPβ LIP isoform was detected at low levels in mammary tumors, however the amount of C/EBPβ LIP detected by Western blot was directly influenced by the protein isolation technique. This suggested that some or all of the C/EBPβ LIP attributed to endogenous production in mammary tumors may be the result of a protein isolation artifact. In contrast to mammary tumors, C/EBPβ LIP could not be detected in protein isolates from normal mammary gland regardless of protein isolation technique. Because C/EBPβ LIP was detectable in mammary tumors and some level of endogenous production (translational or proteolytic) could not be completely ruled out, a second objective was carried out to investigate the effects of C/EBPβ LIP overexpression on cultured mouse mammary epithelial cells. C/EBPβ LIP overexpressing cells exhibited a modest delay in the G_0/G_1 to S phase progression, but no difference in overall cell cycle progression compared to control cell lines. In addition, ectopic C/EBPβ LIP over expression inhibited mouse mammary epithelial cell differentiation (β-casein mRNA expression) by a cell matrix independent mechanism.

METHODS

Mammary Tumors

Weanling female MMTV/c-neu transgenic mice (FVB background) and nontransgenic, age matched controls (FVB) were purchased from Charles River Laboratories, Wilmington, MA. Mice were individually housed in a temperature, light, and humidity-controlled animal facility. Mice were palpated biweekly for 200 days. Mice were killed seven days after tumors were detected by palpation.

Cell Culture

The nontransformed HC 11 mouse mammary epithelial cell line (gift from Dr. Wolfgang Doppler, Universitat Innsbruck, Austria) was cultured in complete growth media consisting of RPMI 1640 (4.5 g/L glucose) supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 10 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 ng/ml Fungizone. COMMA D mouse mammary epithelial cells (gift from Dr. Dan Medina, Baylor University College of Medicine, Houston TX) were cultured in growth media consisting of DMEM supplemented with 10% fetal bovine serum, 10 ng/ml epidermal growth factor, 10 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 ng/ml Fungizone. All medium components were purchased from Life Technologies, Inc., Rockville, MD.

Generation of C/EBPβ LAP and LIP Expressing HC11 Cell Lines

The full length C/EBPβ LAP cDNA (gift from Dr. Steven McKnight, UT Southwestern Medical School, Dallas, TX) was excised from the Bluescript vector (EcoR1/Xho digestion) and ligated into pcDNA3 (Invitrogen, Carlsbad, CA). A truncated C/EBPβ LIP (Nco1/Xho1 digestion fragment from the full-length C/EBPβ LAP cDNA) was excised from Bluescript, filled in, and ligated into pcDNA3 (Invitrogen, Carlsbad, CA). HC11 cells were transfected with the pcDNA3 C/EBPβ LAP, pcDNA3 C/EBPβ LIP, and the pcDNA3 vector control using Transfectam (Promega, Madison, WI). Single clones were selected after culturing in growth media containing 400 μg/ml of Geneticin (Gibco Life Technologies, Rockville, MD). Multiple clonal cell lines were isolated from each transfection treatment and analyzed for C/EBPβ LAP and LIP expression levels by Western blot. Clones expressing the highest levels of C/EBPβ LAP or LIP were selected for further study.

Northern Blot Analysis

Total RNA was isolated using RNAzol B (Tel Test, Inc., Friendswood, TX). Thirty microgram of total RNA was analyzed as previously described [O'Rourke et al., 1997]. The following ^{32}P labeled cDNAs were used as probes: C/EBPα, C/EBPβ, C/EBPδ (gifts from Dr. Steven McKnight, UT Southwestern Medical School, Dallas, TX), CHOP10 (gift from Dr. David Ron, NYU, New York NY), β-casein (gift from Dr. Jeffrey Rosen, Baylor College of Medicine, Houston, TX), and cyclophilin. Densitometry analysis was performed with the AlphaImager 2000 Documentation & Analysis System software (Alpha Innotech, San Leandro, CA).

Flow Cytometry

Cells were harvested and fixed in pre-chilled (−20°C) 70% ethanol for 30 min. After washing (2X with cold PBS) and RNase treatment (1 U of DNase-free RNase for 30 min) 100 μl of propidium iodide (0.5 mg/ml; Boehringer Mannheim, Indianapolis, IN) was added to each sample. Samples were stored at +4°C, protected from light for at least 2 h before flow cytometric analysis.

Growth Rate Determinations

Cells (10^3) were plated in individual wells in a 96-well plate. After 24 h (t = 0), the relative number of viable cells was assessed using the CellTiter 96 aqueous cell proliferation kit (Promega, Madison, WI). Cell monolayers were then rinsed once in serum-free medium and subsequently cultured in complete growth medium or growth arrest medium consisting of RPMI 1640 and 0.1% fetal bovine serum. Samples were taken at 24, 48, and 72 h after the medium switch.

Western Blot Analysis

To obtain whole cell lysates murine mammary tumor tissues were mechanically disrupted in liquid nitrogen and placed in a RIPA buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40. 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, 1X Complete Protease Inhibitors (Boehringer Mannheim, Indianapolis, IN). In addition, the following kinase and phosphatase inhibitors were added: 100 mM NaF, 100 mM $NaVO_3$, 100 mM Na_2MnO4, and 1 μM okadaic acid. Aliquots of these lysates containing 100 μg of protein were subjected to electrophoresis on 12.5% denaturing SDS polyacrylamide gels and then transferred to supported nitrocelulose membranes (Life Technologies, Gaithersburg, MD) at 250 mA for 2 h. Blots were blocked for 60 min in PBST (1X PBS and 0.5% Tween 20) containing 10% non-fat dry milk. They were then incubated for 60 min in PBST containing 5% non-fat dry milk and primary anti-mouse C/EBPβ antisera (1:1,000) (Santa Cruz Biochemicals, Inc., Santa Cruz, CA). Blots were washed in PBST, incubated with anti-rabbit IgG (H&L) HRP-linked secondary antibodies (1:1,000) (New England Biolabs, Beverly, MA) and visualized using enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL). For cytoplasmic and nuclear protein isolations, murine mammary tumor tissues were mechanically disrupted in liquid nitrogen and subjected to Dignam buffers. Cytoplasmic buffer: 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, 1X Complete Protease, 100 mM NaF, 100 mM $NaVO_3$, 100 mM Na_2MnO_4, and 1 μM okadaic acid. Nuclear buffer: 20 mM HEPES (7.9), 1.5 mM $MgCl_2$, 0.42 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1X Complete Protease Inhibitors, 100 mM NaF, 100 mM $NaVO_3$, 100 mM Na_2MnO_4, and 1 μM okadaic acid. Cytoplasmic and nuclear lysates containing 50 μg of protein were

analyzed as described for whole cell protein preparations.

Differentiation Protocol

Cells were grown to confluence and maintained for two days in epidermal growth factor-free RPMI 1640 medium supplemented with 2% fetal bovine serum, 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 ng/ml Fungizone. 5 μg/ml ovine prolactin (Sigma, St. Louis, MO) and 0.1 μM dexamethasone (Sigma, St. Louis, MO) were added to the medium and cells were cultured for an additional four days. Control samples were also isolated after four days, but in the absence of prolactin and dexamethasone. E-C-L Cell Attachment Matrix (entactin-collagen IV-laminin, Upstate Biotechnology, Inc., Lake Placid, New York) was mixed with medium before cells were added. Differentiation was performed as described above.

Growth Arrest Experiments

HC11 cells were grown to 80% confluence, washed with serum free media and cultured in media supplemented with 0.1 % FBS (growth arrest media, GAM). At the indicated times, cell were harvested for Northern or Western blot analysis.

Densitometry and Statistical Analysis

Quantitation of Western blot ECL film results were performed with Alpha Imager software (Alpha Innotech, San Leandro, CA). C/EBPβ LAP and LIP values obtained from densitometric scans were converted to ratios and analyzed by the Mann Whitney U two sample *t*-test.

RESULTS

C/EBPα, C/EBPβ, C/EBPδ, and CHOP10 mRNA levels were assessed in mouse mammary tumors, lactating and involuting mammary glands, and liver. C/EBPα mRNA is detectable at low levels in about 30% of the mammary tumors derived from MMTV/c-neu mice (Fig. 1). Consistent with previous studies in our laboratory, C/EBPα mRNA is virtually undetectable in lactating and involuting mammary gland; however, C/EBPα mRNA is present at relatively high levels in adult liver [Gigliotti and DeWille, 1998]. In contrast to C/EBPα, C/EBPβ mRNA levels are relatively high in all 18 mammary tumors assessed in this study. C/EBPβ mRNA levels in normal mammary tissue (lactating and involuting mammary gland) was about 2–5-fold lower than the C/EBPβ mRNA levels in mammary tumors. C/EBPβ mRNA levels were relatively low in liver.

About 50% of mammary tumors express detectable levels of C/EBPδ mRNA. The C/EBPδ mRNA content of two tumors (11%) approximated the level in the involuting mammary gland. This observation is of interest as C/EBPδ gene expression is highly induced (25–50-fold) during mammary gland involution, which is characterized by massive programmed cell death in the epithelial cell compartment [Quarrie et al., 1996; Gigliotti and DeWille, 1998]. C/EBPδ mRNA levels are low in the liver. CHOP10 mRNA, which is induced during cell

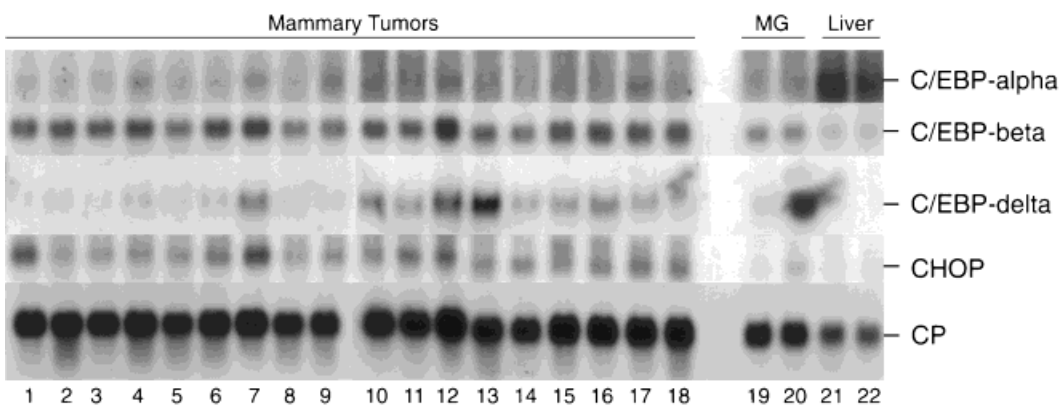


Fig. 1. Northern blot analysis of C/EBP isoform mRNA levels. Total RNA was isolated from mammary tumors, **(lanes 1–18)**; MG = mammary gland, lactating **(lane 19)**; and involuting **(lane 20)**. Liver RNA samples were isolated from the same dams, i.e., liver, lactating **(lane 21)**; liver, involuting **(lane 22)**. All mammary tumors were isolated from MMTV/c-neu transgenic mice (FVB background). Normal mammary and liver samples were from FVB controls. Blots were sequentially probed with the indicated ^{32}P-labelled cDNA probes. CHOP = C/EBP Homologous Protein; CP = cyclophilin (loading control).

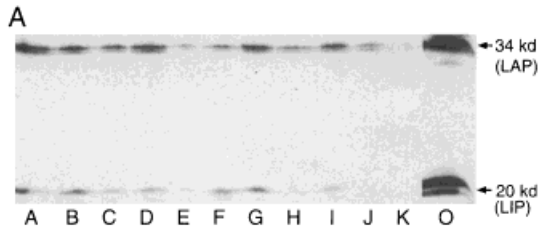
stress [Ron and Habener, 1992], is detectable in most of the mammary tumors tested. Detectable levels of CHOP10 mRNA levels are also found in the involuting mammary gland, but are relatively low in the normal lactating mammary gland and liver.

Mammary Tumor C/EBPβ LAP and LIP Isoform Levels are Influenced by Protein Isolation Technique

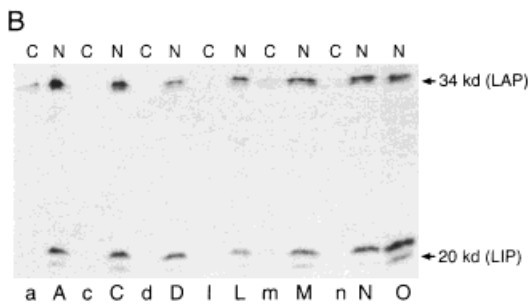
Mammary tumor C/EBPβ LAP and LIP levels were assessed by Western Blot analysis using antisera against the carboxyl terminal region of the C/EBPβ protein. A broad spectrum protease inhibitor cocktail was included in all protein isolation protocols (see Materials and Methods). When protein isolations were carried out using a rapid whole cell isolation technique, the mean mammary tumor C/EBPβ LAP:LIP ratio was slightly over 5:1 (5.3:1, Fig. 2A). When mammary tumor isolations are carried out using a multi-step nuclear/cytoplasmic fractionation protocol, nearly all the detectable C/EBPβ LAP and LIP was localized to the nucleus and the LAP:LIP ratio decreased significantly to 1.6:1 ($P < 0.05$). This decrease occurred despite the presence of multiple protease inhibitors (Fig. 2B). Sufficient tumor mass was available from three tumors ("A", "C", and "D") to carry out Western blot analysis with lysates obtained from both the rapid whole cell and the multi-step nuclear/cytoplasmic fractionation protocols. The average C/EBPβ LAP: LIP ratio for tumors A, C, and D isolated by the rapid whole cell protein isolation protocol was ~6:1. In contrast, the average C/EBPβ LAP: LIP ratio for tumors A, C, and D isolated by the nuclear/cytoplasmic fractionation protocol was ~1:1 ($P < 0.05$). These results demonstrate a significant reduction in C/EBPβ LAP:LIP ratios following nuclear/cytoplasmic fractionation. It is important to note that the difference in C/EBPβ LAP:LIP ratios between isolation protocols is not due to preferential translocation or accumulation of either isoform in the nucleus as both C/EBPβ LAP and LIP are localized almost exclusively to the nucleus [O'Rourke et al., 1997 and Fig. 2B].

Mouse Mammary Gland C/EBPβ LAP Levels are Elevated During Gestation-LIP is Undetectable

Mammary gland epithelial cells undergo intense proliferation during gestation. Previous studies have reported that C/EBPβ LAP levels are elevated relative to C/EBPβ LIP (LAP:LIP ratio; <5:1) during gestational proliferation in the rat mammary gland [Raught et al., 1995]. We investigated mouse mammary gland



Tumor	LAP Level	LIP Level	LAP/LIP Ratio
A	148,580	96,140	1.6/1.0
B	121,486	54,188	2.2/1.0
C	83,030	7,866	10.6/1.0
D	117,116	19,228	6.1/1.0
E	24,472	4,370	5.6/1.0
F	47,196	23,598	2.0/1.0
G	119,738	47,198	2.5/1.0
H	34,086	5,244	6.5/1.0
I	107,502	8,740	12.3/1.0
J	33,212	4,370	7.6/1.0
K	5,244	4,370	1.2/1.0



Tumor	LAP Level	LIP Level	LAP/LIP Ratio
A	123,728	92,796	1.3/1.0
C	40,700	52,096	0.8/1.0
D	106,634	86,284	1.2/1.0
L	73,260	25,234	2.9/1.0
M	78,144	46,398	1.7/1.0
N	94,424	61,864	1.5/1.0

Fig. 2. Western blot analysis of C/EBPβ LAP and LIP isoforms in mammary tumors from MMTV/c-neu transgenic mice. **A:** C/EBPβ LAP and LIP isoforms were analyzed in mammary tumor whole cell lysates **(lanes A–K)**. C/EBPβ LAP and LIP control **(lane "O")**. **B:** Mammary tumors (A, C, D, L, M, N) were fractionated into cytoplasmic (c; lower case letter) and nuclear (n; upper case letter) compartments. C/EBPβ LAP and LIP control (lane "O"). Lysates (50 μg/lane) were separated by 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane. Membranes were probed with an anti mouse C/EBPβ primary antibody (Santa Cruz Biotechnology, Polo Alto CA) and a horseradish peroxidase-conjugated secondary antibody. Detection was by the ECL system.

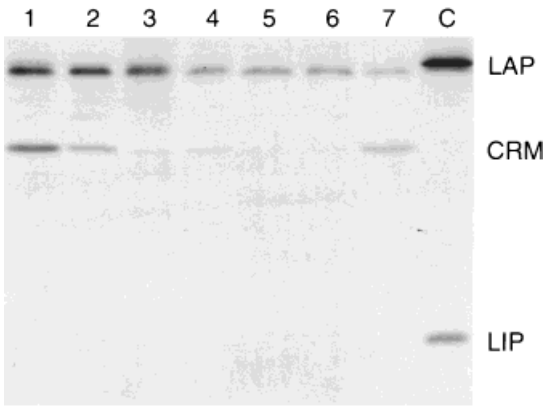


Fig. 3. Western blot analysis of C/EBPβ LAP and LIP isoforms during mouse mammary gland lactation time course. Mammary gland (inguinal) nuclear lysates were isolated at various times throughout the lactation time course. **Lane 1:** mid pregnant; **lane 2:** late pregnant; **lane 3:** lactation Day 1; **lane 4:** lactation Day 10; **lane 5:** 24 h involuting; **lane 6:** 48 h involuting; **lane 7:** 120 h involuting; **lane 8:** LAP over expressing cell line. CRM = cross-reacting material. Lysates (50 μg/lane) were separated by 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane. Membranes were probed with an anti mouse C/EBPβ primary antibody (Santa Cruz Biotechnology, Polo Alto CA) and a horseradish peroxidase-conjugated secondary antibody. Detection was by the ECL system.

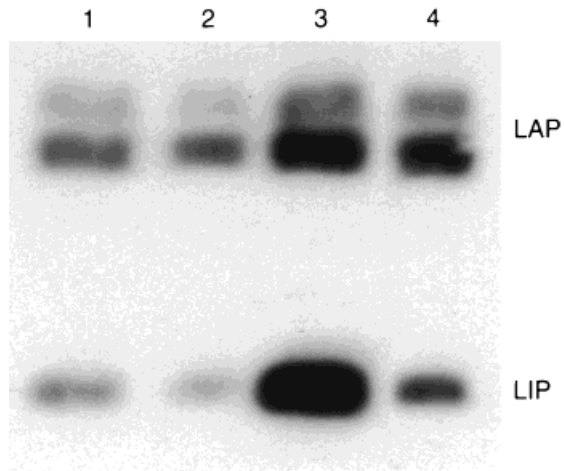


Fig. 4. Western blot analysis of C/EBPβ LAP and LIP levels in HC11 whole cell lysates. **Lanes 1:** nontransfected HC11 parental cells; **lanes 2:** vector transfected HC11 cell (PC); **lanes 3:** C/EBPβ LIP transfected HC11 cells; **lanes 4:** C/EBPβ LAP transfected HC11 cells. Lysates (50 μg/lane) were separated by 12.5% SDS-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane. Membranes were probed with an anti mouse C/EBPβ primary antibody (Santa Cruz Biotechnology, Polo Alto, CA) and a horseradish peroxidase-conjugated secondary antibody. Detection was by the ECL system.

C/EBPβ LAP and LIP content during gestation, lactation and involution using nuclear/cytoplasm protein isolation techniques (including complete protease inhibitors). Mammary gland C/EBPβ LAP levels were elevated during gestation and in early lactation compared with lactation and involution time points (Fig. 3). In contrast, C/EBPβ LIP was undetectable during gestational proliferation, lactation, or involution in the normal mouse mammary gland (Fig. 3).

C/EBPβ LAP and LIP Levels in Stably Transfected Nontransformed Mouse Mammary Epithelial Cells (HC11)

Although mammary tumor C/EBPβ LIP levels can be influenced by isolation technique, C/EBPβ LIP was detected in mammary tumors using our most stringent lysate isolation protocol (Fig. 2). The detection of C/EBPβ LIP in mammary tumors, coupled with the absence of C/EBPβ LIP in normal mammary gland, suggests that C/EBPβ LIP could be present in mammary tumors in vivo. The presence of C/EBPβ LIP in preneoplastic mammary epithelial cells could facilitate transformation by influencing mammary epithelial cell growth control or differentiation. To investigate the role of C/EBPβ isoforms in nontransformed mammary epithelial cells, C/EBPβ LAP and LIP over expressing HC11 cell lines were established. C/EBPβ LAP and LIP levels were assessed by Western Blot and quantitated by densitometry in whole cell protein lysates from the following HC11 cell lines: HC11 parental, HC11 vector transfected control (PC), and HC11/C/EBPβ LIP (L2) and HC11/C/EBPβ LAP (B6) over expressing cell lines. C/EBPβ LAP and LIP levels were similar between parental HC 11 cells and vector transfected control (PC) cells (Fig. 4). The C/EBPβ LIP transfected HC11 cell line (L2) (lane 3) contained four times the level of C/EBPβ LIP and approximately twice the level of LAP compared to parental HC11 and PC control cells. The C/EBPβ LAP transfected HC11 cell line (B6) (lane 4) contained ~50% higher C/EBPβ LAP and LIP levels compared to control cell lines. In the C/EBPβ LAP overexpressing B6 cell line the increased C/EBPβ LIP levels may be due to proteolytic cleavage of the overexpressed LAP isoform.

The Influence of Ectopic C/EBPβ LIP and LAP Expression on HC11 Cell Proliferation

Increased mammary gland C/EBPβ LAP levels have been reported during gestational

proliferation and increased C/EBPβ LIP levels have reported in proliferating nontransformed and transformed mouse mammary epithelial cells [Raught et al., 1996]. The influence of C/EBPβ LAP and LIP on HC11 mammary epithelial cell proliferation was assessed in HC11 cell lines stably transfected with C/EBPβ LAP and LIP expression vectors and compared with HC11 nontransfected (parental) cells and PC (vector transfected) control HC11 cells. Proliferation of the HC11 parental cells was higher than the HC11 PC controls and both LAP and LIP over expressing cell lines in the first 24 h growth interval (approximate HC11 doubling time) after cell plating. There were no significant differences, however, in proliferation between the parental HC11, HC11 vector transfected (PC) controls, C/EBPβ LAP over expressing (B6), or C/EBPβ LIP over expressing (L2) cell lines at any of the subsequent 24 h growth intervals (Fig. 5A). These results suggest that ectopic expression of either C/EBPβ LIP or LAP does not influence proliferation of nontransformed mammary epithelial cells under optimal growth conditions.

The capacity of cells to grow in the absence of serum and growth factors has been used as an indicator of cell transformation status. To investigate the influence of C/EBPβ LIP and LAP over expression on growth in the absence of serum and growth factors, sub-confluent HC11 cells were cultured in growth arrest media (0.1% FBS). Compared with results from complete growth media experiments (Fig. 5A), proliferation in the absence of serum and growth factors was significantly impaired in all cell lines at all time points. In the initial 24 h, both the HC11 parental and C/EBPβ LAP over expressing cell lines exhibited a 1.6-fold increase in cell number. There was essentially no change in cell number in the PC control cell line. The number of C/EBPβ LIP overexpressing cells decreased slightly in the first 24 h after plating in the absence of serum and growth factors (Fig. 5B). There were no significant differences in proliferation between the parental HC11, vector transfected HC11 (PC) controls, C/EBPβ LAP over expressing (B6) or LIP overexpressing (L2) cell lines at any of the subsequent 24 h intervals (Fig. 5B). These results demonstrate that the withdrawal of serum and growth factors dramatically reduces HC11 cell growth, regardless of C/EBPβ LAP or LIP expression levels. C/EBPβ LIP over expression modestly reduced initial growth rates, however as length of time in culture increased, over expression of C/EBPβ LAP or LIP had no effect on cell growth in serum and growth factor free media.

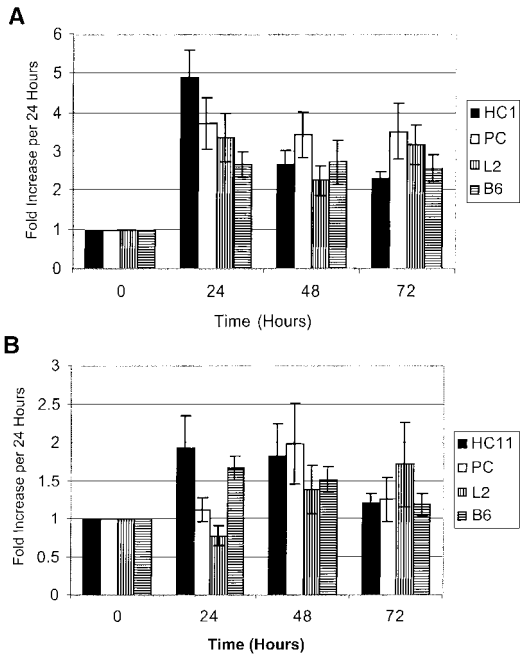


Fig. 5. The influence of ectopic C/EBPβ LIP and LAP expression on HC11 cell proliferation in complete growth media and growth arrest media. **A:** Growth rates (cell numbers) of HC11, vector transfected HC11 controls (PC), C/EBPβ LIP over expressing HC11 (L2) and C/EBPβ LAP over expressing HC11 (B6) cell lines were plated at low density (10^3 /well) in 96-well plates in complete growth media. **B:** Growth rates (cell numbers) of HC11, vector transfected HC11 controls (PC), C/EBPβ LIP over expressing HC11 (L2), and C/EBPβ LAP over expressing HC11 (B6) cell lines were plated at low density (10^3 / well) in 96-well plates in growth arrest media. Cell numbers were assessed 0, 24, 48, and 72 h after plating by the CellTiter 96 aqueous cell proliferation assay kit (Promega, Madison, WI). Results are mean values of three wells/time point and are representative of three experiments. Error bars represent standard errors of the mean.

The Influence of C/EBPβ LAP and LIP Over Expression on HC11 Cell Cycle Progression

Overall growth rates of HC11 cells under optimal and suboptimal growth conditions were generally unaltered by C/EBPβ LAP and LIP overexpression (Fig. 5). Modest alterations in the cell cycle progression, however, may not alter overall growth rates, but could alter in cell cycle checkpoint efficiency and eventually influence cell phenotype [Ohtsubo and Roberts, 1993]. To investigate the influence of C/EBPβ LAP and LIP over expression on cell cycle

T=0 Hr.	G0/G1	S	G2+M
HC11	88.88	7.44	3.68
PC	94.54	3.48	1.98
L2	92.89	3.83	3.28
B6	94.53	3.18	2.3
T=14 Hr.	G0/G1	S	G2+M
HC11	46.36	53.64	0.0
PC	39.88	60.12	0.0
L2	85.13	14.87	0.0
B6	61.95	38.05	0.0
T=16 Hr.	G0/G1	S	G2+M
HC11	26.67	73.33	0.0
PC	34.95	63.8	1.25
L2	51.19	48.81	0.0
B6	23.14	76.86	0.0
T=18 Hr.	G0/G1	S	G2+M
HC11	26.22	69.47	4.3
PC	20.23	66.16	13.62
L2	22.15	77.47	0.38
B6	8.6	72.3	19.1
T=22 Hr.	G0/G1	S	G2+M
HC11	50.65	26.79	22.56
PC	39.72	28.83	31.45
L2	17.48	34.05	48.47
B6	41.73	24.78	33.48

Fig. 6. Cell cycle analysis of HC11, vector transfected HC11 controls (PC), C/EBPβ LIP over expressing HC11 (L2) and C/EBPβ LAP over expressing HC11 (B6) cell lines. All cell lines were initially plated at 80% confluence. After 48 h in growth arrest media (T = 0), complete growth media was added to initiate a synchronous cell cycle. At the indicated time points (T = 0, 14, 16, 18, and 22) cells were harvested, stained with propidium iodide and analyzed by a fluorescence-activated cell sorter. The results are representative of three independent experiments.

progression, confluent cell cultures were maintained in growth arrest media (0.1% FBS) for 72 h. Flow cytometry indicated that growth arrest was attained in all cell lines (88–94% of cells in G_0/G_1) (Fig. 6, T = 0). Fourteen hours after the addition of complete growth media and the initiation of a synchronous cell cycle, 53% of HC11 parental and 60% of the vector transfected HC11 cells (PC) had entered S phase. Approximately 38% of C/EBPβ LAP over expressing cells had entered S phase by 14 h. In contrast, only about 15% of C/EBPβ LIP over expressing cells had progressed from G_0/G_1 to S phase by 14 h. By 16 h, the percentage of HC11 parental, PC, and B6 cells in S phase was within a relatively tight range (64–77%), but the C/EBPβ LIP overexpressing cell line (L2) continued to lag behind in S phase entry (49% of cells in S phase). By 18–22 h after cell cycle initiation the percent cells in S phase was roughly equivalent among all cell lines. These results demonstrate that C/EBPβ LIP over expression induced a consistent 2–4 h delay in G_0/G_1 to S phase progression. However, C/EBPβ LIP and LAP overexpression did not dramatically alter HC11 cell cycle progression.

Ectopic C/EBPβ LAP Enhances HC11 Differentiation (β-Casein Expression)-Ectopic C/EBPβ LIP Expression Inhibits HC11 Differentiation (β-Casein Expression)

Previous reports have demonstrated that ectopic C/EBPβ LIP expression blocks 3T3-L1 differentiation [Yeh et al., 1995]. The influence of C/EBPβ LIP and LAP overexpression on mammary epithelial cell differentiation was assessed by Northern Blot analysis, using β-casein mRNA expression as the differentiation marker. HC11, PC control, and LAP over expressing (B6) cell lines expressed β-casein mRNA after four days of lactogenic hormone exposure (Fig. 7). Interestingly, C/EBPβ LAP over expressing mammary epithelial cells (B6 cells) expressed β-casein mRNA at ~2-fold higher levels than both HC11 and PC control cell lines. In contrast, β-casein mRNA was undetectable in C/EBPβ LIP over expressing (L2) cells after four days of lactogenic hormone exposure. These results indicate that C/EBPβ LAP overexpression increases β-casein expression compared to controls. In contrast, C/EBPβ LIP overexpression inhibits β-casein mRNA expression in mammary epithelial cells.

Previous studies have shown that an extracellular matrix (ECM) is required for mammary epithelial cell differentiation in vitro [Lelievre et al., 1996; Lin et al., 1993]. To investigate the possibility that C/EBPβ LIP overexpressing cells were defective in ECM production an artificial extracellular matrix (ECL) was added to all culture dishes prior to cell plating and exposure to lactogenic hormones. HC11, PC control and B6 cell lines expressed β-casein

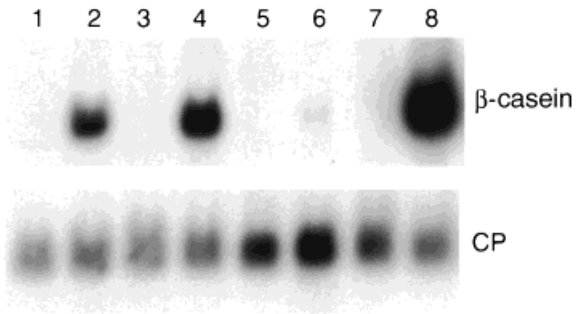


Fig. 7. The influence of ectopic C/EBPβ LIP and LAP expression on HC11 cell differentiation (β casein mRNA expression) on plastic. Total RNA was isolated after four days in culture in the presence **(lanes 2, 4, 6, and 8)** or absence **(lanes 1, 3, 5, and 7)** of lactogenic hormones. The results demonstrate the capacity of each cell line to differentiate in response to lactogenic hormones. Lanes 2: HC11; lane 4: PC controls; lane 6: C/EBPβ LIP over expressing HC11 cells (L2); lane 8: C/EBPβ LAP over expressing HC11 (B6) cells. Undifferentiated controls were cultured in the absence of lactogenic hormones. Undifferentiated controls are presented in lanes 1: HC11; lane 3: PC controls; lane 5: C/EBPβ LIP over expressing HC11 cells (L2); lane 7: C/EBPβ LAP over expressing HC11 (B6) cells. Blots were probed with ^{32} P-labelled β casein and cyclophilin (CP) cDNA probes. CP was used as a loading control. Results are representative of three independent experiments.

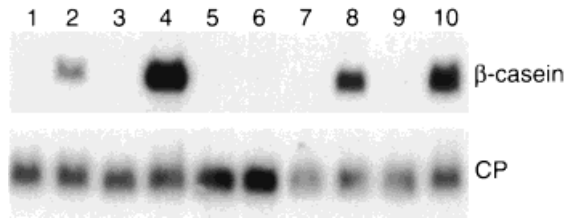


Fig. 8. The influence of ectopic C/EBPβ LIP and LAP expression on HC11 cell differentiation (β casein mRNA expression) on ECM. Total RNA was isolated after four days in culture on E-C-L (entactin-collagen IV-laminin) attachment matrix in the presence (or absence) of lactogenic hormones. The results demonstrate the capacity of each cell line to differentiate in response to lactogenic hormones. **Lane 2:** HC11; **lane 4:** PC controls; **lane 6:** C/EBPβ LIP over expressing HC11 cells (L2); **lane 8:** C/EBPβ LAP over expressing HC11 (B6) cells; **Lane 10:** (10) COMMA D mouse mammary epithelial cells (positive control for functioning E-C-L matrix; COMMA D cells will not differentiate without the presence of and E-C-L matrix).Undifferentiated controls were cultured in the absence of lactogenic hormones. Undifferentiated controls are presented in **lane 1:** HC11; **lane 3:** PC controls; **lane 5:** C/EBPβ LIP over expressing HC11 cells (L2); **lane 7:** C/EBPβ LAP over expressing HC11 (B6) cells; **lane 9:** COMMA D cells. Blots were probed with ^{32} P-labelled β casein and cyclophilin (CP) cDNA probes. CP was used as a loading control. Results are representative of three independent experiments.

mRNA when cultured on the ECL matrix and exposed to lactogenic hormones for four days (Fig. 8). The COMMA D mouse mammary epithelial cell line, which is defective in ECM production and requires an artificial ECM to differentiate was included as a control. Elevated levels of β-casein mRNA were detected in COMMA D cells, indicating that the artificial ECM was functional in this assay. Despite the presence of a functional ECM, β-casein mRNA was undetectable in L2 cells after exposure to lactogenic hormone treatment. These results indicate that ectopic C/EBPβ LIP expression inhibits β-casein mRNA expression, a marker for mammary epithelial cell differentiation, by a mechanism that does not involve the ECM.

DISCUSSION

The first question addressed in this study was the relative expression of C/EBPs in mouse mammary gland and mammary tumors. C/EBPβ is the only C/EBP family member that is constitutively expressed at relatively high levels in lactating and involuting mammary gland and also in 100% (18/18) of mouse mammary tumors from MMTV/c-neu transgenic mice. Previous reports have demonstrated an essential role for C/EBPβ in mammary gland development and function [Robinson et al., 1998; Seagroves et al., 1998]. These results suggest that C/EBPβ may retain a biological role in nontransformed and transformed cells of mammary origin. Although the significance of the increased C/EBPβ gene expression in mammary tumors is unknown, identification of genes expressed in mammary tumors in MMTV/c-neu transgenic mice may be particularly relevant to human breast cancer. The human homologue of c-neu, ErbB2, is over expressed in about 25% of breast cancer patients and 75% of breast cancer patients with metastasis [Rowse et al., 1998].

Variable levels of mRNAs encoding other C/EBP family members (C/EBPα, C/EBPδ, and CHOP) were also detected in mammary tumors. C/EBPδ is known to play a role in mammary epithelial cell growth arrest and apoptosis [O'Rourke et al., 1997, 1999; Gigliotti and DeWille, 1998; Gigliotti et al., 1999; Hutt et al., 2000]. The presence of elevated C/EBPδ mRNA levels is indicative of tumors with a significant number of cells entering, or attempting to enter G_0 growth arrest and/or apoptosis. The presence of elevated levels of the stress-inducible CHOP mRNA suggests that some

tumors may be undergoing "stress" from hypoxia, glucose deprivation, or the activation of cell death programs [Ron and Habener, 1992].

It has been previously reported that both C/EBPβ LAP and C/EBPβ LIP are induced in the rodent mammary gland during gestation [Raught et al., 1995; Rosen et al., 1998]. Our results confirmed that C/EBPβ LAP levels are elevated in mouse mammary gland during gestation and early lactation, then decline throughout the remainder of lactation and early involution. This suggests a primary role for C/EBPβ LAP in the early proliferation phase of mammary epithelial cell commitment to differentiation. In contrast to previous reports [Raught et al., 1995; Rosen et al., 1998], we did not detect elevated levels of C/EBPβ LIP in normal mammary gland during gestation. In our studies C/EBPβ LIP was virtually undetectable in the mammary gland throughout gestation, lactation, and involution. These results suggest that C/EBPβ LIP does not play an essential role in the normal mouse mammary gland during the lactational time course. In addition, these results raise questions regarding the origin and relevant physiological function of C/EBPβ LIP in mammary gland biology.

Early reports indicated that a "leaky ribosome" mechanism was responsible for C/EBPβ LIP production [Descombes and Schibler, 1991], however, limited experimental data exists to support this hypothesis. Recent work by Lincoln et al. suggests that most, if not all, of the C/EBPβ LIP detected by Western blot is the result of inadvertent proteolysis of C/EBPβ LAP during protein isolation, not "leaky ribosome" alternate translation initiation. [Baer et al., 1998; Lincoln et al., 1998; Baer and Johnson, 2000]. Initial mammary gland and mammary tumor protein isolations carried out in our lab used published whole cell lysis methods that did not include a broad spectrum of serine, cysteine, and calpain protease inhibitors. These isolations resulted in the detection of significant amounts of C/EBPβ LIP. Mammary gland C/EBPβ LAP:LIP ratios of 5:1 and mammary tumor LAP:LIP ratios as low as 1:3 were observed when protein lysate isolations were carried out without the appropriate protease inhibitors [DeWille et al., 1997]. In the present study, the inclusion of lysis buffers containing a more complete cocktail of protease inhibitors significantly reduced C/EBPβ LIP levels. In addition, techniques such as nuclear/cytoplasmic separations, which require more extensive sample manipulation, appear to lower C/EBPβ LAP:LIP ratios when compared with rapid whole cell isolations. These results suggest that a significant proportion of immunodetected C/EBPβ LIP is the result of isolation-induced proteolysis of C/EBPβ LAP. Lincoln et al. have also demonstrated that the 14 kD C/EBPβ isoform is generated by isolation-induced proteolysis of C/EBPβ LAP [Baer et al., 1998]. Although our results indicate that the C/EBPβ LAP:LIP ratio is influenced by the cell lysate preparation protocol, some C/EBPβ LIP is still detected in mammary tumors. It is possible that some C/EBPβ LIP is present in mammary tumors as a result of endogenous proteolytic cleavage of C/EBPβ LAP. Alternatively, it is also possible that even rapid isolation methods using complete protease inhibitors cannot completely eliminate some artifactual C/EBPβ LAP proteolysis and LIP production during isolation.

Ectopic over expression of C/EBPβ LIP increased HC11 LIP levels 4-fold and also appeared to increase endogenous C/EBPβ LAP levels ~2-fold (Fig. 4). Similar results have also been recently reported by Raught et al. in C/EBPβ LIP over expressing mouse mammary gland and cultured mammary epithelial cells [Zahnow et al., 2001]. Although the mechanism in which C/EBPβ LIP could increase C/EBPβ LAP levels is unknown, C/EBPβ LIP forms dimers with C/EBPβ LAP. It is possible that the formation of C/EBPβ LIP/C/EBPβ LAP dimers may stabilize C/EBPβ LAP, resulting in an increase in C/EBPβ LAP steady state protein levels. Alternatively, C/EBPβ LIP functions as a dominant negative transcriptional inhibitor when complexed with other C/EBP family members and bZIP proteins. Elevated C/EBPβ LIP levels could reduce the transcription of genes that encode proteases or other protein products that function in the cleavage or degradation of C/EBPβ LAP. Although C/EBPβ LIP dominant negative function has been described in a number of reports, at least one report has shown increased transcriptional activation in experimental assays containing increasing amounts of C/EBPβ LIP [Hsieh et al., 1998]. This suggests that elevated C/EBPβ LIP levels could increase transcription of genes that may enhance the transcription, translation, or stabilization of C/EBPβ LAP. Finally, C/EBPβ LIP over expression does not result in an increase in C/EBPβ LAP levels in all cell types.

Ectopic over expression of C/EBPβ LIP in COS and 3T3-L1 cells did not increase C/EBPβ LAP levels [Ossipow et al., 1993; Yeh et al., 1995]. These results suggest that ectopic over expression of C/EBPβ LIP increases C/EBPβ LAP steady state protein levels by a mechanism that may be specific to mammary epithelial cells.

Our results suggest a primary role for C/EBPβ LAP in the early proliferation phase of mammary epithelial cell commitment to differentiation. Although we detected elevated LAP levels in vivo in mammary gland isolates during gestation, we did not detect an increase in proliferation rates in mammary epithelial cells over expressing C/EBPβ LAP in vitro. Similarly, the ectopic over expression of C/EBPβ LIP in HC11 mammary epithelial cells did not have a significant influence on proliferation. In fact, C/EBPβ LIP over expressing mammary epithelial cell numbers decreased slightly in the initial 24 h after plating in media lacking serum and growth factors. This suggests that C/EBPβ LIP over expression may inhibit initial growth rates (G_0/G_1 to S phase progression). Since cell growth (cell number) at 48 and 72 h after plating did not differ among the cell lines these results indicate that ectopic over expression of C/EBPβ LAP or LIP does not promote proliferation of HC11 mammary epithelial cells.

Although over expression of C/EBPβ LAP and LIP isoforms did not alter HC11 mammary epithelial cell proliferation, C/EBPβ LAP or LIP interaction with Rb could alter restriction point control and influence G_0/G_1 to S phase progression. Over expression of gene products that function in G_1, such as c-myc, cyclin D1, and cyclin E shorten G_0/G_1 to S phase progression [Ohtsubo and Roberts, 1993; Quelle et al., 1993; Arber et al., 1997; Dang, 1999]. In c-myc and cyclin D1 over expressing cell lines this shortens the overall cell cycle length and reduces doubling time [Ohtsubo and Roberts, 1993; Quelle et al., 1993; Arber et al., 1997; Dang, 1999]. In cyclin E overexpressing cells however, shortening of G_1 is compensated for by a lengthening of S phase, resulting in no change in cell cycle length [Ohtsubo and Roberts, 1993]. In our studies C/EBPβ LIP over expression consistently prolonged G_0/G_1 to S phase progression by 2–4 h. Despite this delay, cell cycle length, doubling time, and over cell growth (cell numbers) of C/EBPβ LIP over expressing cells was similar to C/EBPβ LAP over expressing HC11 cells and HC11 control cell lines. These results suggest that C/EBPβ LIP over expression prolongs G_0/G_1 to S phase progression, but this is compensated for by shortening of S phase. Although it is conceivable that changes in cell cycle phase length could influence the sequential expression of cell cycle regulated genes or the timing or fidelity of DNA replication, the significance of these observations is unknown.

In addition to growth, C/EBPβ LAP and LIP also influence cellular differentiation. Ectopic over expression of C/EBPβ LAP induces 3T3-L1 differentiation, even in the absence of adipogenic hormone treatment [Yeh et al., 1995]. In contrast, C/EBPβ LIP over expression inhibits 3T3-L1 differentiation [Yeh et al., 1995]. After exposure to lactogenic hormones, C/EBPβ LAP over expressing cells (B6) express approximately twice as much β-casein mRNA compared to the parental HC11 and vector (PC) controls. This apparent enhancement of HC11 differentiation is similar to a previous report investigating the influence of C/EBPβ LAP over expression on 3T3-L1 cell differentiation [Yeh et al., 1995]. It suggests that C/EBPβ LAP exerts a general enhancing influence on cell differentiation programs. Alternatively, since the β-casein gene promoter contains multiple C/EBP consensus sites it is possible that C/EBPβ LAP over expression may directly transactivate the β-casein gene promoter [Doppler et al., 1995]. Although this is possible, results presented in this report (Fig. 3) and in previous reports consistently show that C/EBPβ LAP levels are significantly lower (as much as 20-fold lower) in the lactating mammary gland compared to the proliferating mammary gland during gestation [Raught et al., 1995]. This indicates that there is no direct relationship between C/EBPβ LAP levels and β-casein gene expression.

In contrast to C/EBPβ LAP over expressing HC11 cells, C/EBPβ LIP over expressing HC11 cells (L2) do not express β-casein, an indicator of mammary epithelial cell differentiation, in response to lactogenic hormone treatment. This result is also similar to previous work from 3T3-L1 cells [Yeh et al., 1995]. It suggests that C/EBPβ LIP over expression inhibits cell diferentiation programs. It is possible that C/EBPβ LIP over expression may inhibit C/EBPβ LAP activation of C/EBP consensus sites in the β-casein gene promoter. However, other gene promoters that contain functional C/EBP consensus sites, such as c-fos, exhibit

apparently normal activation and expression (data not shown). Alternatively, ectopic C/EBPβ LIP expression could interfere with LAP interactions with Rb or other regulatory proteins in differentiation. The inability of C/EBPβ LIP over expressing cells to express the β-casein differentiation marker was not reversed by culturing L2 cells on an ECM. This indicates that the C/EBPβ LIP associated defect in mammary epithelial cell differentiation does not directly involve the ECM.

In summary, these results support an important, but complex role for C/EBPβ in mammary gland and mammary epithelial cell biology. Current studies are aimed at further defining the role of C/EBP LAP and LIP in mammary gland biology and tumorigenesis.

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REFERENCES

Arber N, Doki Y, Han E, Sgambato A, Zhou P, Kim N, Delohery T, Klein MG, Holt PR, Weinstein IB. 1997. Antisense to cyclin D1 inhibits the growth and tumorigenicity of human colon cancer cells. Cancer Res 57:1569–1574.

Baer M, Williams SC, Dillner A, Schwartz RC, Johnson PF. 1998. Autocrine signals control CCAAT/enhancer binding protein β expression, localization, and activity in macrophages. Blood 92:4353–4365.

Baer M, Johnson PF. 2000. Generation of truncated C/EBP isoforms by in vitro proteolysis. J Biol Chem 275:26582–26590.

Cao Z, Umek RM, McKnight SL. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538–1552.

Chen P-L, Riley D, Chen Y, Lee W-H. 1996a. Retinoblastoma protein positively regulates terminal adipocyte differentiation thorough direct interaction with C/EBPs. Genes &Dev 10:2794–2804.

Chen P-L, Riley D, Chen-Kiang S, Lee W. 1996b. Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. Proc Natl Acad Sci 93:465–469.

Croninger C, Leahy P, Reschef L, Hanson RW. 1998. C/EBP and the control of phosphoenolpyruvate carboxykinase gene transcription in the liver. J Biol Chem 273:3162–31632.

Dang CV. 1999. c-myc target genes involved in cell growth, apoptosis, and metabolism. Mol Cell Biol 19:1–11.

Descombes P, Schibler U. 1991. A liver-enriched transcriptional activator protein, LAP, and transcriptional inhibitory protein, LIP, are translated from the same mRNA. Cell 67:569–579.

DeWille JW, Sattler A, Lee J, Yuan R. 1997. US Army Breast Cancer Research Program 1,506-W.

Doppler W, Welte T, Philipp S. 1995. CCAAT/Enhancer-binding protein isoforms β and δ are expressed in mammary epithelial cells and bind to multiple sites in the β-casein gene promoter. J. Biol Chem 270:17962–17969.

Gigliotti AP, DeWille J. 1998. Lactation status influences expression of CCAAT/enhancer binding protein isoform mRNA in the mouse mammary gland. J Cell Physiol 174:232–239.

Gigliotti AP, DeWille JW. 1999. Local signals induce CCAAT/enhancer binding protein-δ (C/EBP-δ) and C/EBP-β mRNA expression in the involuting mouse mammary gland. Breast Cancer Research and Treatment 59:57–63.

Greenbaum LE, Li W, Cressman DE, Peng Y, Ciliberto G, Poli V, Taub R. 1998. CCAAT enhancer-binding protein beta is required for normal hepatocyte proliferation in mice after partial hepatectomy. J Clin Invest 102:996–107.

Hsieh CC, Xiong W, Xie Q, Rabek JP, Scott SG, An MR, Reisner PD, Kuninger DT, Papaconstantinou J. 1998. Effects of age on the posttranscriptional regulation of CCAAT/enhancer binding protein α and CCAAT/enhancer binding protein β isoform synthesis in control and LPS-treated livers. Mol Biol Cell 9:1479–1494.

Hurst HC. 1994. Transcription factors. 1: bZIP proteins. Protein Profile 1(2):123–168.

Hutt JA, O'Rourke JP, DeWille JW. 2000. Signal transducer and activator of transcription 3 activates CCAAT enhancer-binding proteinδ gene transcription in G_0 growth-arrested mouse mammary epithelial cells and in involuting mouse mammary gland. J Biol Chem 275:29123–29131.

Lekstrom-Himes J, Xanthopoulos KG. 1998. Biological role of CCAAT/enhancer binding protein family of transcription factors. J Biol Chem 273:28545–28548.

Lelievre S, Weaver VM, Bissell MJ. 1996. Extracellular matrix signaling from the cellular membrane skeleton to the nuclear skeleton: a model of gene regulation. Recent Prog Hor Research 51:417–432.

Lin CQ, Bissell MJ. 1993. Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J 7:737–743.

Lincoln AJ, Monczak Y, Williams SC, Johnson PF. 1998. Inhibition of CCAAT/enhancer-binding protein α and β translation by upstream open reading frames. J Biol Chem 273:9552–9560.

O'Rourke J, Yuan R, DeWille J. 1997. CCAAT/enhancer-binding protein-δ (C/EBP-δ) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem 272:6291–6296.

O'Rourke J, Newbound G, Hutt J, DeWille J. 1999. CCAAT/enhancer-binding protein δ regulates mammary epithelial cell G_0 growth arrest and apoptosis. J Biol Chem 274:16582–16589.

Ohtsubo M, Roberts JM. 1993. Cyclin-dependent regulation of G_1 in mammalian fibroblasts. Science 259:1908–1912.

Ossipow V, Descombes P, Schibler U. 1993. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. Proc Natl Acad Sci 90:8219–8223.

Quarrie LH, Addey CV, Wilde CJ. 1996. Programmed cell death during mammary tissue involution induced by

weaning, litter, removal, and milk stasis. J Cell Phys 168:559–569.

Quelle DE, Ashmun RA, Shurtleff SA, Kato J, Bar-Sagi D, Roussel MF, Sherr CJ. 1993. Overexpression of mouse D-type cyclins accelerates G_1 phase in rodent fibroblasts. Genes & Dev 7:1559–1571.

Raught B, Liao WSL, Rosen JM. 1995. Developmentally and hormonally regulated CCAAT/enhancer-binding protein isoforms influence β-casein gene expression. Mol Endocrinol 9:1223–1232.

Raught B, Gingras AC, James A, Medina D, Sonenberg N, Rosen JM. 1996. Expression of a translationally regulated dominant-negative CCAAT/enhancer-binding protein β isoform and up-regulation of the eukaryotic translation initiation factor 2α are correlated with neoplastic transformation of mammary epithelial cells. Cancer Res 56:4382–4386.

Robinson GW, Johnson PF, Hennighausen L, Sterneck E. 1998. The C/EBPβ transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes & Dev 12:1907–1916.

Ron D, Habener J. 1992. CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 6:439–453.

Rosen JM, Zahnow C, Kazansky A, Raught B. 1998. Composite response elements mediate hormonal and developmental regulation of milk protein gene expression. Biochem Soc Symp 63:101–113.

Rowse GJ, Ritland SR, Gendler SJ. 1998. Genetic modulation of neu proto-oncogene-induced mammary tumorigenesis. Cancer Res 58:2675–2679.

Sabatakos G, Davies GE, Grosse M, Cryer A, Ramji DP. 1998. Expression of the genes encoding CCAAT-enhancer binding protein isoforms in the mouse mammary gland during lactation and involution. Biochem J 334:205–210.

Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM. 1998. C/EBPβ, but not C/EBPα, is essential for ductal morphogenesis lobuloalveolar proliferation and functional differentiation in the mouse mammary gland. Genes & Dev 12:1917–1928.

Sealy L, Malone D, Pawlak M. 1997. Regulation of the *cfos* serum response element by C/EBPβ. Mol Cell Biol 17:1744–1755.

Tang QQ, Lang MD. 1998. Activation and centromeric localization of CCAAT/enhancer binding proteins during the mitotic clonal expansion of adipocyte differentiation. Genes and Devel 13:2231–2241.

Welm A, Timchenko N, Darlington G. 1999. C/EBPα regulates generation of C/EBPβ isoforms through activation of specific proteolytic cleavage. Mol Cell Biol 19:1695–1704.

Williams SC, Cantwell CA, Johnson PF. 1991. A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. Genes Dev 5:1553–1567.

Wu Z, Xie Y, Bucher NL, Farmer SR. 1995. Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPAR gamma and stimulates adipogenesis. Genes Dev 9:2350–2363.

Yeh WC, Cao Z, Classon M, McKnight SL. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes & Dev 9:168–181.

Zahnow CA, Younes P, Lauicia R, Rosen JM. 1997. Overexpression of C/EBPβ- LIP, a naturally occurring, dominant-negative transcription factor, in human breast cancer. J Nat Canc Inst 89:1887–1891.

Zahnow CA, Cardiff RD, Laucirica R, Medina D, Rosen JM. 2001. A role for CCAAT/enhancer binding protein β-liver-enriched inhibitory protein in mammary epithelial cell proliferation. Cancer Res 61:261–269.