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The objective of this fellowship is to characterize the roles which C/EBPα and C/EBPβ play in normal mammary gland development and breast cancer. In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells. LIP can act as a dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to excessive cellular proliferation. Overexpression of C/EBPβ–LIP in the mammary gland of pregnant and lactating transgenic mice results in an abnormal phenotype characterized by enlarged ducts, and regions of hyperplasia. Additionally, preliminary experiments suggest that overexpression of LIP in TM3 mouse mammary cells doubles the growth rate. These observations are consistent with the hypothesis that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression.

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Cynthia C. Zahnow 8/10/98

PI - Signature Date

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## INTRODUCTION

The failure of a cell to maintain balance between proliferation and terminal differentiation often results in tumor formation. The C/EBP family of DNA binding proteins plays a pivotal role in maintaining this balance by regulating the expression of genes involved in terminal differentiation (1, 11, 12). In general, C/EBPα is predominantly expressed at high levels in terminally differentiated, growth arrested cells and the C/EBPβ isoforms, most notably LIP, are expressed at high levels in actively proliferating cells . LIP can act as a dominant negative isoform, which when dimerized with other C/EBP family members suppresses transcriptional activity. Because of an increased DNA affinity of the LIP isoform, this inhibition can occur even at sub-stoichiometric ratios of LIP/LAP (3). Consequently, an increase in LIP levels may inhibit terminal differentiation and lead to excessive cellular proliferation. Consistent with this hypothesis, we have observed elevated LIP levels in several different mouse mammary tumors. These data, as well as the reported observation that C/EBPβ can directly interact with the retinoblastoma protein, has prompted us to investigate the role of C/EBP in mammary gland tumorigenesis (2, 7). We plan to determine whether the overexpression of LIP in both mammary cell lines and in transgenic mice can alter cell growth and facilitate hyperplasia or tumorigenesis. Additionally, we will investigate the mechanisms by which hormonal factors are involved in this overexpression and how elevated levels of LIP may influence the transactivation potential of other C/EBP family members. We hypothesize that overexpression of LIP in mice may block terminal differentiation, and help facilitate uncontrolled proliferation and tumorigenesis. Finally, we will employ a novel combination of gene knockout and mammary gland transplantation technology to study the role of C/EBPα in regulating terminal differentiation.

The following specific tasks were proposed for the 36 months of this proposal. Due to the availability of mice, reagents and/or experimental difficulty, a few tasks were completed earlier than expected and some will take additional time.

**Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis.** Months 1-24. This requires 150 mice.

**Task 1:** Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP. (Months 1-6).

**Task 2:** Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice. (Months 6-12).

**Task 3:** Construction of the WAP-LIP-WAP construct. (Months 1-6).

**Task 4:** Generation and screening of transgenic mice which overexpress LIP. (Months 6-18).

**Task 5:** Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice. (Months 12-36).

**Technical Objective 2: Determination of the effects of lactogenic hormones on C/EBPβ isoform expression, post-translational processing and functional activity in a mouse mammary epithelial cell line (HC 11).** Months 1-12.

**Task 6:** Generation of a stably transfected mouse mammary cell line (HC 11) with a multimeric C/EBP promoter-CAT construct and treatment with lactogenic hormones. (Months 1-6).

**Task 7:** Analysis of C/EBPβ expression and activity in response to hormonal treatment. (Months 1-6).

**Task 8:** Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing. (Months 6-18).

**Task 9:** Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio. (Months 18-24).

**Technical Objective 3: Analysis of the roles of C/EBP proteins on mammary gland development in a C/EBPα knockout mouse.** Months 12-36. This requires 48 mice.

**Task 10:** Removal and transplantation of mammary glands from recipient C/EBPα mice into host 129 mice. (Months 12-24).

**Task 11:** Analysis of the development of mammary glands from C/EBPα knockout mice. (Months 24-36).

# BODY

## Experimental Methods

### Protein Extraction and Western Blot Analysis

Tissue and/or cells were disrupted in RIPA buffer (50mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% desoxycholate, 150 mM NaCl, 1 mM EGTA, 0.2% SDS) containing the following kinase, phosphatase and protease inhibitors; 1 mM $NaVO_3$, 1 mM NaF, 1 mM $Na_2MoO_4$, 10 nM okadaic acid, and 1μg/ml benzamidine, aprotinin, soybean trypsin inhibitor and antipain. Aliquots of these lysates containing 100 μg of protein were electrophoresed on denaturing SDS 12%-polyacrylamide mini-gels, then transferred to PVDF membranes (Millipore, Bedford, MA) overnight at 75 mA. Blots were blocked 90 min in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Tween-20) containing 3% non-fat dry milk (Carnation, Glendale, CA) then incubated for 90 min in this solution containing antibodies (0.5 ng/ml) (Santa Cruz) prepared against C/EBPβ. Blot washes consisted of TBST (without milk) three times for 5-10 min each, with agitation. Blots were then incubated for 60 min in blocking solution containing 200 ng/ml biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, England) and washed. Lastly, blots were incubated for 30 min in blocking solution containing 40 ng/ml streptavidin-horseradish peroxidase (Oncogene Science, Uniondale, NY) and washed as before. Enhanced chemiluminesence (Hyperfilm, Amersham) and chemifluoresence reagents (Storm Fluoroimager from Molecular Dynamics) were used for visualization as per the manufacturer's instructions.

### Immunohistochemistry

Tissues were fixed for 6 hrs in 10% neutral-buffered formalin (NBF), embedded, sectioned, deparaffinized through a graded series of xylenes and alcohols and then rehydrated in water and phosphate-buffered saline. Antigens were retrieved by boiling for 10 min in 2 M urea, and endoperoxidases were blocked in a methanol solution containing 3% $H_2O_2$ for 15 min at room temperature. After washing, tissue was incubated for 1 hr at room temperature with 10% normal

goat serum (NGS) (Sigma), and 20% avidin blocking solution (Vector Laboratories, Inc.) in 1X phosphate-buffered saline and 0.1% tween 20 (PBST). Excess blocking solution was drained and polyclonal rabbit primary antibody for C/EBPβ (1:300, Santa Cruz Biochemicals, Inc.) containing 20% biotin blocking solution (Vector Laboratories, Inc.) in 10% normal goat serum (NGS) was applied overnight at 4°C. Biotin-conjugated goat anti-rabbit secondary antibody at 1:200 dilution in 10% NGS was incubated for 30 min at room temperature. Biotin-avidin binding and DAB detection were carried out according to the manufacturer's instructions (Vector Laboratories, Inc.). Control slides were treated identically except that 6 μg of C/EBPβ peptide (Santa Cruz Biochemicals, Inc.) were incubated with the primary antibody for 15-30 min at 0°C.

**Detection of Cellular Proliferation**

Bromodeoxyuridine (BrdU) incorporation was used to label cells which have entered S phase. Evaluation of the number of mammary epithelial cells with BrdU incorporation aided in the determination of the level of cellular proliferation. Mice were anesthetized with 0.5-1.0 ml of Avertin and mammary glands were surgically removed 2 hrs after injection of 5-bromo-2'deoxyuridine (BrdU, *s.c.*, 0.3 mg/10 g body weight). Tissue was fixed in 10% neutral buffered saline for 6 hrs, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized through a graded series of xylenes and alcohols and then rehydrated in water and phosphate-buffered saline. Non-specific binding of tissue was blocked in 3% bovine serum albumin in 1X PBST for 15 min at 37°C and then rinsed in PBS for 1 min at room temperature. AntiBrdU containing nuclease (Amersham) was applied and sections were incubated for 90 min at 37°C. After three 1 min washes in PBST the peroxidase anti-mouse IgG was applied and sections were incubated for 30 min at 37°C washed as before and visualized using DAB-nickel according to the manufacturer's instructions (Vector Laboratories, Inc.).

**Detection of Apoptosis (TUNEL)**

Tissue was fixed in 10% neutral buffered saline for 6 hrs, embedded in paraffin, and sectioned at 5 μm. Sections were deparaffinized as described above and incubated with proteinase K (1 μg/ml) for 10 min at 37°C. Sections were washed 3X in PBST and blocked in PBS containing 10% MeOH, 0.3% $H_2O_2$ for 20 min at room temperature, washed in PBS as before and incubated with an end labeling mixture containing terminal deoxynucleotidyltransferase (TdT) buffer (30 mM Tris, pH 7.2, 140 mM NaCacodylate and 1 mM cobalt chloride), 1 nmol biotinylated dUTP, and 20 U of TdT for 60 min at 37°C. Labeled sections were incubated with ABC reagent according to the manufacturer's instructions and then incubated with DAB-nickel according to the manufacturer's instructions (Vector Laboratories, Inc.).

**Transfection of TM3 cells**

TM 3 cells were grown and maintained using HEPES buffered D-MEM/F-12 growth media containing: 2% fetal bovine serum, 10 μg/ml insulin, L-glutamine, 5 ng/ml epidermal growth factor (EGF), and 5 μg/ml gentamycin sulfate. Cells were transfected with three different transfection reagents, Lipofectamine (Gibco, BRL), the Perfect Transfection series of pfx lipids (Invitrogen) and Superfect (Qiagen). It was determined that Superfect yielded the least toxicity and best transfection efficiency. At 20 to 40% of confluence, cells were stably transfected with the DNA of interest using Superfect (Qiagen). Stably transfected cells were cloned and maintained with 0.2 mg G418 per ml growth media.

## Results and Discussion

**Technical Objective 1: Generation of two complementary models in which we can study the effects of LIP overexpression on mammary gland development and tumorigenesis.** Months 1-24.

**Task 1: Generation of a stably transfected mouse mammary (TM 3) cell line which overexpresses LIP.** (Months 1-6).

To initiate these studies, several inherent difficulties involved in the selection, transfection and maintenance of mammary cells had to be overcome. In general, mammary cells are difficult to transfect. and this technique had to be optimized. Cells were transfected with three different transfection reagents, Lipofectamine (Gibco, BRL), the Perfect Transfection series of pfx lipids (Invitrogen) and Superfect (Qiagen). Of these Superfect yielded the least toxicity and best transfection efficiency and was chosen for all future transfections.. An additional concern was choosing a cell line with low basal levels of LIP in order to clearly assess changes due to the overexpression of LIP. There are few mammary cell lines available which are considered normal or non-tumorigenic. We assayed LIP protein levels in two "normal" mouse lines, (HC 11 and TM 3) and a "normal" human line (MCF-10A). The TM 3 line contained the lowest levels of basal LIP expression and was thus used in our experiments. TM 3 cells are density dependent and hence take a very long time to expand when grown at the low density of single colonies on a large plate. Consequently we had to transfect in 12 well plates and slowly expand the clones . Clonal transfectants were more desirable as opposed to transfected pools because the experimental results would be difficult to interpret unless all the cells expressed LIP at the same level and responded in a similar manner. The type of promoter used to drive expression of LIP is also quite critical to these experiments. Because LIP acts as a dominant negative, a promoter containing C/EBP sites could be negatively regulated by the production of LIP and LIP levels would eventually decline. We chose to work with the cytomegalovirus immediate-early enhancer/promoter which usually gives strong constitutive expression in many cell types and is not known to contain any C/EBP consensus sites.

### Two plasmid Tet-On Gene Expression System

To more clearly study the effects of LIP overexpression on the cell cycle we transfected LIP into TM 3 cells as a regulatable system. Initially we chose Bujard and Gossen's two plasmid tetracycline regulatable system (4, 5). In the Tet-On system, expression of LIP should be activated in the presence of doxycycline. An immediate drawback to this system is that the cells are established as a double-stable cell line from two consecutive rounds of transfection, selection, cloning and screening. By necessity, the cells undergo many cell divisions and these later passage cells could be very different from the earlier passages. A total of 31 clones went through the first round of transfection with the transactivator plasmid pUHD172-1neo and were then cotransfected with the tetracycline responsive plasmid pUHD10-3-LIP and the hygromycin selection vector pTK-Hyg. Each of the resultant double-stable clones were treated with or without doxycycline and tested via Western blot analysis for the production of doxycycline regulatable LIP protein. LIP was elevated in three of the six clones tested, but the expression was not regulatable. We believe the elevated LIP expression observed was a result of either: leaky expression due to integration site differences or a change in protein expression profiles because of the multiple cellular divisions.

### Retroviral Tetracyline System Vectors

To circumvent the problems of double-stable selection, poor transfection efficiency and integration site difficulties we decided to forgo the two plasmid based system and use a retroviral based approach. Retroviral tet vectors integrate at a single site and express both the activator protein and the gene of interest. Stable cell lines are thus developed with only a single infection

and screening. However, the retroviral vector must first be transfected into a packaging cell line and stable virus producing cells can then be selected. An improved tet retroviral system has been constructed by Dr. Helen Blau's laboratory at Stanford, which she has agreed to send us as soon as her manuscript describing this system has been accepted for publication. Unfortunately, due to delays in the review process this has taken over six months and she only recently said the construct should be forthcoming. Consequently, the retroviral experiments are on hold until we receive the retroviral construct.

**CMV Driven Expression Plasmids**

In addition to using the inducible expression systems, LIP was cloned into a plasmid containing the cytomegalovirus immediate-early enhancer/promoter which usually gives strong constitutive expression in many cell types. CMV-LIP / pSV2-neo and pSV2-neo (as control) were transfected into TM 3 cells and stable clones were selected and screened for LIP expression with Western Blot analysis. Our results demonstrated that none of the CMV-LIP/pSV2-neo clones contained elevated LIP expression. We suspected that the lack of LIP expression was due to a problem with promoter activity in the absence of a 5' intron. Consequently, treatment with 1.5 mM sodium butyrate, which inhibits histone deacetylase, facilitated promoter activity and elevated LIP expression levels. Two of the highest CMV-LIP/ pSV2-neo expressing clones and two pSV2-neo clones with low basal levels of LIP were chosen to test whether LIP overexpression affects the growth rate of these cells. The clones were plated in triplicate, grown for 17 days in the presence of sodium butyrate and cell numbers were analyzed with a coulter counter. The slopes of the growth curves for the two CMV-LIP/ pSV2-neo clones was approximately twice that of the pSV2-neo clones. These data demonstrate that LIP overexpression in mouse mammary epithelial cells can result in an increased growth rate.

Because sodium butyrate may elicit non-selective effects on cellular transcription we wanted to repeat these experiments in the absence of sodium butyrate treatment. Accordingly, the LIP expression cassette had to be re-cloned into a vector with a strong promoter which would not requrie sodium butyrate activation. We chose pCI-neo (Promega) because it contains both a chimeric intron downstream of the CMV enhancer/promoter and the SV40 enhancer and early promoter region upstream of the neomycin phosphotransferase gene. Transfection studies have demonstrated that introns which are 5' to the inserted cDNA often increase the level of gene expression. TM 3 cells have been transfected with pCIneo-LIP or pCIneo and these clones are currently being selected and slowly expanded. If this construct produces a sufficient level of LIP expression, then the growth rate and cell cycle characteristics of these transfectants will be examined.

**Task 2: Transplantation of LIP overexpressing TM 3 cells into the fat pad of BALB/c mice.** (Months 6-12).

This task has not been initiated because despite the original published reports, transfected TM 3 cells appear to have lost the ability to grow out and form normal ducts in cleared mammary fat pad of syngeneic mice (Dr. Daniel Medina, personal communication). There appears to be a general problem of genetic stability in many of these established mammary cell lines. Consequently, primary mammary epithelial cultures infected with retroviral vectors may provide the only viable alternative. Experiments to test the utility of this technique are currently underway in our laboratory using retroviruses containing both GFP and epitope-tagged proteins.

**Task 3: Construction of the WAP-LIP-WAP construct.** (Months 1-6).
**(Completed see Progress Report 1997).**

**Task 4: Generation and screening of transgenic mice which overexpress LIP.** (Months 6-18).
**(Completed see Progress Report 1997).**

**Task 5: Analysis of LIP overexpression on mammary gland development in both transplanted BALB/c mice and transgenic mice.** (Months 12-36).

Although the analysis of the LIP phenotype is ongoing, some preliminary results have been obtained.

Observations of mice which are over two years in age and have had multiple litters, have demonstrated that this protein has no affect on the ability of the mice to nurse their young. Litter sizes and pup weights appear to be normal. In addition, preliminary results obtained by Western blot analysis, have demonstrated that β-casein levels are unchanged in pregnant and lactating glands of transgenic mice.

We have repeatedly bred several females in order to maintain the LIP expression level and facilitate any neoplastic changes that might occur. However, after approximately one and a half years of breeding no tumors have been observed. However, hyperplastic regions were observed in the involuted glands of mice which were approximately two years old. These regions appear to be composed of epithelial cells (hyperplastic alveolar nodule) and resemble an enlarged lobule which normally is not present during involution. We are currently generating age matched FVB controls to determine if the hyperplastic tissue is a normal occurrence in aged (1.5 to 2 year), multiply bred FVB mice.

Examination of hematoxylin/eosin stained 5μm tissue sections have demonstrated that mice which overexpress LIP in their mammary glands have an altered or abnormal phenotype. In 10 day lactating tissue, the alveoli appear to be smaller or more condensed than in control animals. (Control mice for all experiments consisted of wild type FVB mice and/or non-transgenic littermates). Additionally, luminal secretions appear to be reduced and abnormal. The shape of many of the alveolar cells is distorted and the cytoplasmic to nuclear ratio may be altered. Additionally, there is an increase in the stromal compartment. This may represent a decrease in the ability of the epithelial cells to completely fill out the mammary fat pad. This phenotype has been observed as early as day 18 of pregnancy and as late as day 10 of lactation. Additionally, abnormally large ducts have been observed in mid-lactating and involuted glands (Fig. 1A and B). Some of these enlarged ducts have regions of intraductal hyperplasia, infolding and fingerlike or pappillary projections which extend into the lumen (Fig 1C and D).

C/EBPβ immunohistochemistry experiments are in progress to determine if these atypical tissues correspond to regions which overexpress the LIP transgene. Using both BrDU immunohistochemistry, and TUNEL we are investigating whether LIP is affecting cellular proliferation or apoptosis in the hyperplastic regions.

To study the function of LIP in a background of reduced C/EBPβ-LAP we have bred the C/EBPβ-LIP mice to mice lacking the C/EBPβ gene. We hypothesized that the phenotype observed in these mice would be more severe than that observed in just the LIP transgenics. Unfortunately, a loss of transgene expression was observed in these offspring. It was determined that C/EBPβ is necessary for regulation of expression of the WAP promoter, which drives LIP transgene expression in our system. Additionally, we also bred the C/EBPβ–LIP mice with transgenic mice expressing mutated p53 ($172^{R-H}$) which also is targeted to the mammary gland with the WAP promoter. These mutated p53 mice show increased susceptibility to mammary carcinogenesis when induced by DMBA and the resulting tumors have increased genomic instability (7). Unfortunately, these tumors take well over a year to develop and all of the 6 bigenics died of unknown causes before development of any mammary tumors.

**Task 6: Generation of a stably transfected mouse mammary cell line (HC 11) with a multimeric C/EBP promoter - CAT construct and treatment with lactogenic hormones.** (Months 1-6).

HC 11 cells were stably transfected with a multimeric C/EBP promoter - CAT construct and treated with lactogenic hormones, insulin and hydrocortisone. C/EBPβ protein levels were

analyzed in these cells to verify that the stable cell line would respond to lactogenic hormones by decreasing C/EBPβ protein levels. However, it was observed that C/EBPβ protein levels remained unchanged in response to hormonal treatment. As described in task 7, C/EBPβ protein levels in non-transfected, early passage HC 11 cells were reduced in response to lactogenic hormones. We have observed that later passage cells (*e.g.*, p20 vs. p9) do not respond to hormones in the same manner as the earlier passage cells. A normal consequence of generating a stable cell line is that the cells undergo many cell divisions and may be very different from the earlier passage cells that were their precursors. Thus, passage number appears to affect the response of HC 11 cells to hormones. Consequently, it is not possible to complete this task in HC 11 cells, but an alternative cell line might be TM 6, or MCF-10A.

**Task 7: Analysis of C/EBPβ expression and activity in response to hormonal treatment.** (Months 1-6).

C/EBPβ expression was analyzed in HC 11 cells which were treated for 5 days with hydrocortisone (1μg/ml). Western blot analysis demonstrated that both the LIP and LAP isoforms were decreased after 1 day of treatment and by 5 days of treatment the reduction was substantial (Fig. 2). The mechanism for this decrease has not been investigated but may be due to both a reduction in transcription of the C/EBPβ gene and/or a reduction in the translation of the individual isoforms.

To investigate whether a translational mechanism was involved in the observed decrease in C/EBPβ protein isoforms, the levels of 4E-BP1, which is the inhibitory binding protein for the cap-binding initiation factor eIF4E, were also examined by Western blot analysis. BP1 levels remained unchanged in response to treatment with glucocorticoids, but decreased as a function of the number of days the cells were at confluence.

**Task 8: Correlation of LAP/LIP levels with transactivation activity and investigation of post-translational processing.** (Months 6-18).

**Task 9: Correlation of the effect of ovarian steroids and prolactin on tumor growth in ovariectomized rats with the LAP/LIP ratio.** (Months 18-24).

A preliminary experiment was conducted to test the effect of ovarian steroids on the LAP/LIP ratio in rats. Mid-pregnant rats were chosen for this experiment because LIP levels are elevated and more easily detected during pregnancy. Mid-pregnant rats were either ovariectomized (ovx) or sham (non-ovx) treated. At the time of ovariectomy hormone replacement was administered in the form of a 20 mg progesterone pellet or a control beeswax pellet placed dorsally in the mid-thoracic region. Three days later the right number 4 mammary gland was removed for protein analysis. Ovariectomy, without hormone replacement, (ovx-control) resulted in elevated LIP levels, thereby decreasing the LAP/LIP ratio (Fig. 3). In contrast, progesterone treatment did not increase LIP expression to the levels that were observed in the ovx-control rats, but instead decreased LAP levels (Fig. 3). Interestingly, ovariectomy also induced the expression of several proteins which are slightly larger than LIP. It is unknown whether these proteins are cross reactive or are C/EBPβ isoforms which have been modified by the process of ovariectomy. The biochemical mechanism and physiological significance of these changes are unknown and remain to be investigated.

In a different experiment, the carcinogen, NMU, was used to produce hormonally responsive mammary gland tumors in rats. When the tumors were of palpable size they were biopsied and LAP/LIP levels were analyzed. Immediately after biopsy, the rats were ovariectomized to initiate tumor regression. Once the tumors had regressed to at least 50% of the initial size, a second biopsy was taken for analysis of LAP/LIP levels. Although, the LAP/LIP ratio was analyzed in seventeen tumors, no consistent pattern in the changes of the LAP/LIP ratio were observed. Using the NMU animal model of tumor growth, there was no correlation of the LAP/LIP ratio with the effect of ovarian steroids on tumor growth.

**Tasks 10 and 11. Removal and transplantation of mammary glands from recipient C/EBPα mice into host 129 mice and analysis of the development of mammary glands from C/EBPα knockout mice.**
(Due to the availability of knockout mice and technical expertise it was necessary to initiate these tasks in year 1 instead of years 2 and 3).
**(Completed see Progress Report 1997).**

Independent studies performed by other members of the laboratory have demonstrated that C/EBPβ, but not C/EBPα is required for ductal morphogenesis, lobuloalveolar development and functional differentiation in the mammary gland (10). The role of C/EBPβ has been confirmed independently by another laboratory in a different mouse strain (9). Thus, these studies reinforce the importance of studying the role of the different C/EBPβ isoforms in normal mammary gland development and breast cancer.

## CONCLUSIONS

Proliferative diseases such as cancer, are often the result of failure to withdraw from the cell cycle at the $G_1$ checkpoint. Multiple signal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late $G_1$ is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). Recent studies (2) have demonstrated that Rb directly interacts with and activates all of the C/EBPβ isoforms; however, it is not known how this interaction affects Rb activity. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland, and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. Support for this hypothesis comes from the observations that the LIP/LAP ratio is regulated during proliferative phases of both liver and mammary gland development (1, 10, 11).

We have proposed that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression. This hypothesis has been supported by our observation that the C/EBPβ protein isoforms, and in particular, the naturally-occurring dominant-negative LIP isoform, have been detected and are highly expressed in aggressive, poorly differentiated infiltrating ductal carcinomas (13; See appendix 1). These studies do not answer the question of whether LIP overexpression, in aggressive tumors, facilitates tumor progression or is simply a result of increased proliferation. However, we now have preliminary data from both tissue culture and transgenic animals which suggests that elevated LIP levels do increase cellular growth rate and can lead to hyperplasia and abnormal mammary gland development. These observations are consistent with the hypothesis that the C/EBPβ-isoforms may play a role in regulating terminal differentiation and cell cycle progression. Additionally, we have determined that the C/EBPβ-isoforms are regulated by lactogenic hormones. LIP/LAP protein levels are decreased by glucocorticoids and ovariectomy increased LIP levels and decreased the LAP/LIP ratio. The biochemical mechanism and physiological significance of these hormonal changes are unknown and remain to be investigated.

We are currently extending our tissue culture studies to examine what effects if any, LIP overexpression has on cell cycle proteins (p21, p27, cyclin D1) and how these changes lead to an increased growth rate in mammary cells. Additionally, in our WAP-LIP-WAP transgenic mice, we plan to investigate the downstream genomic targets of LIP overexpression and how these genes contribute to the altered mammary phenotype observed. These studies will be crucial to understanding the role that the C/EBPβ-isoforms play in breast cancer.

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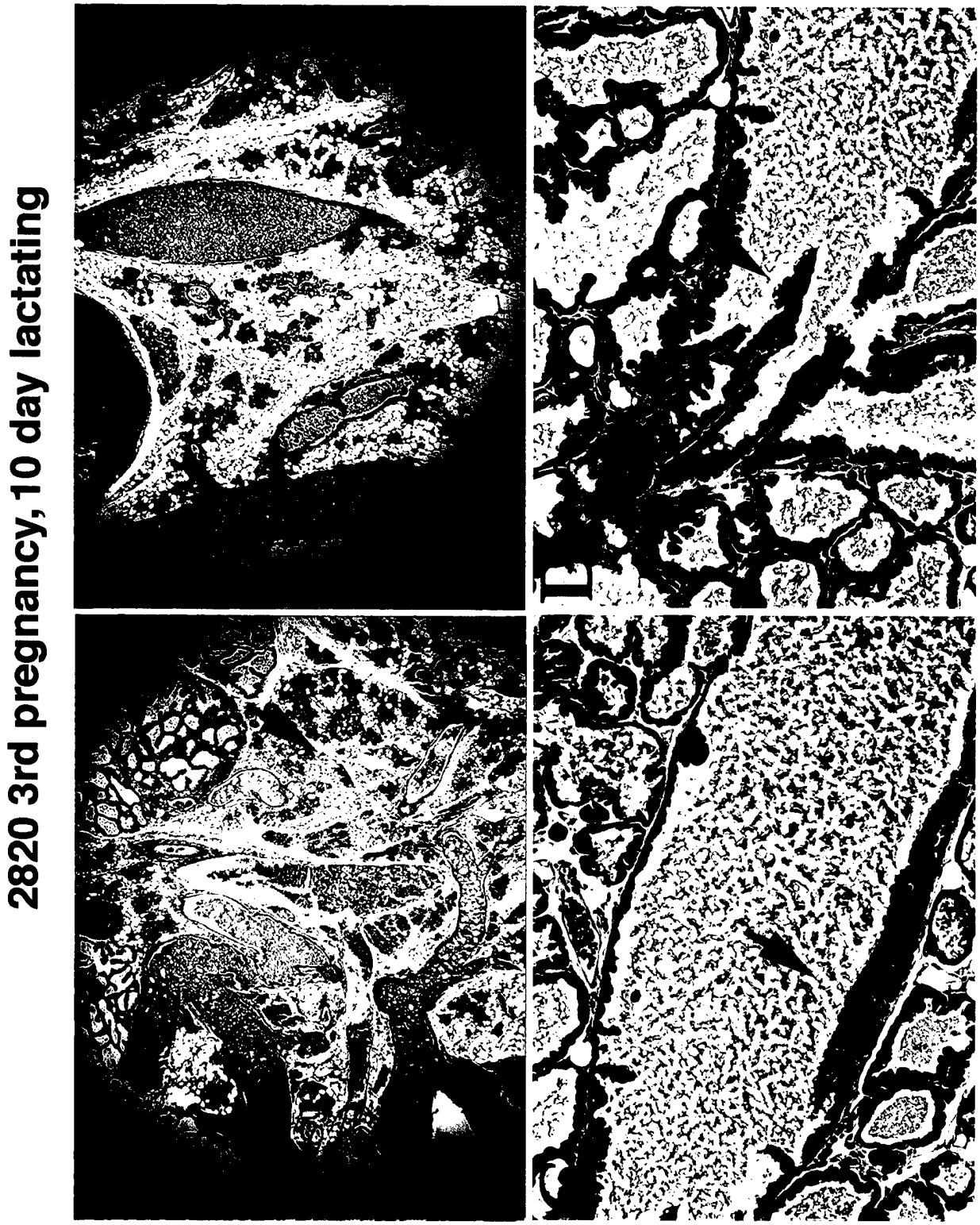
## FIGURE LEGENDS

**Figure 1.** Hematoxylin and eosin stained (5 μm) sections of mammary glands from day 10 of lactation. Panels A and B are both lower magnification (4x) to show the abnormally large size and frequency of the ducts in this representative mammary gland. The arrow in (A) points to a lobule or group of lobules which are underdeveloped and represent a mid-pregnant developmental stage. Surrounding this underdeveloped region are distended alveoli which are more normal in appearance. (C). Higher magnification (40x) of an enlarged duct showing a hyperplastic region which is composed of multiple layers of epithelial cells. (D). Enlarged duct with intraductal fingerlike or papillary projections.

**Figure 2.** Effects of glucocorticoid treatment on LAP/LIP levels in HC 11 cells as analyzed by Western blot analysis. Cells were plated in duplicate and grown at confluence for 3 days before treatment with hydrocortisone (H, 1μg/ml). The + control lane is a TM 3 cell line which has been transfected with LIP. LAP/LIP levels start to decrease at day 1 of treatment and continue to day 5.

**Figure 3.** Effects of ovariectomy on LAP/LIP ratios in mid-pregnant rats as analyzed by Western blot analysis. Mid-pregnant rats were either ovariectomized (ovx) or sham treated (2 rats). At the time of ovariectomy, hormone replacement was administered in the form of a 20 mg progesterone pellet (3 rats) or a control (2 rats) beeswax pellet placed dorsally in the mid-thoracic region. The lane labeled TGFα represents extracts from mammary gland tumors generated in mice which overexpress the transgene TGFα. Note the occurrence in the ovx lanes of several proteins with a lower mobility or higher molecular weight than LIP.

Figure 1.



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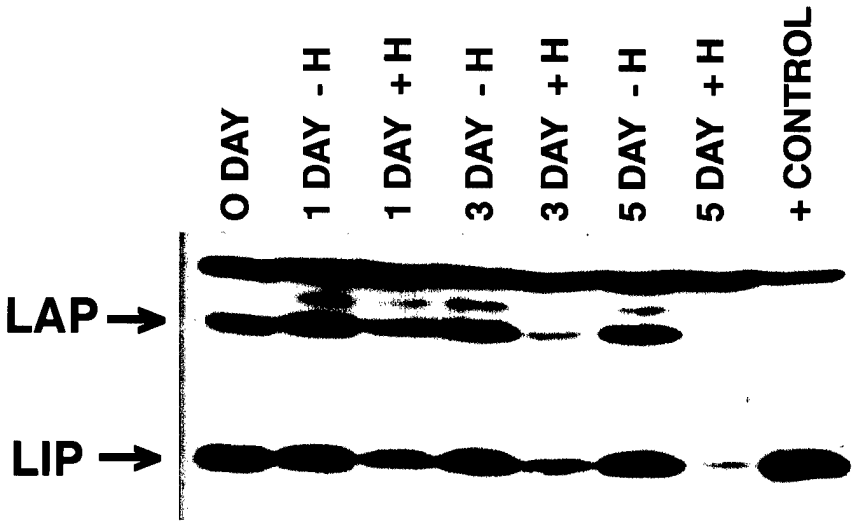
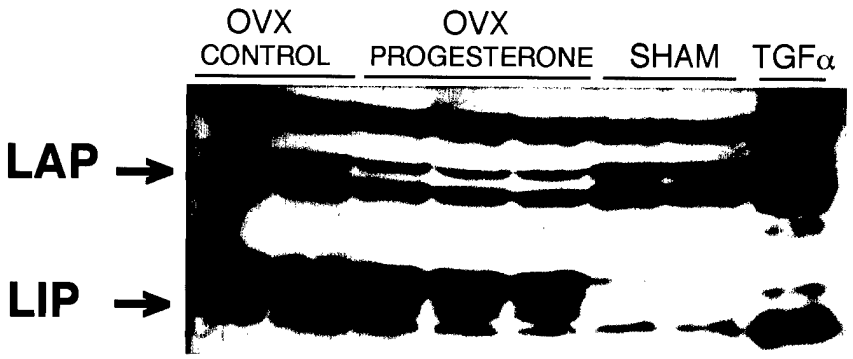


Figure 3.



# Overexpression of C/EBPβ-LIP, a Naturally Occurring, Dominant-Negative Transcription Factor, in Human Breast Cancer

*Cynthia A. Zahnow, Pamela Younes, Rodolfo Laucirica, Jeffrey M. Rosen**

***Background:*** **When cells fail to maintain a balance between proliferation, terminal differentiation, and programmed cell death, cancer often results. The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors regulates many genes involved in the processes of proliferation and terminal differentiation. The messenger RNA for C/EBPβ is translated into two major isoforms, LAP (liver-enriched activating protein) and LIP (liver-enriched inhibitory protein). LIP levels appear to be elevated in mouse mammary tumors but not in hyperplastic mammary tissues. We tested whether LIP expression is elevated in human breast cancer and whether elevated expression is associated with biologic predictors of the aggressiveness of the disease.** ***Methods:*** **Homogenates of infiltrating ductal carcinoma specimens from 39 women were analyzed for C/EBPβ protein content by western blot analysis, and the ratio of LAP to LIP in specimens containing high levels of LIP (i.e., levels approximately 15 times higher than those in tumor specimens that express little or no LIP) was also determined. Nonparametric statistical analyses were performed.** ***Results:*** **LIP was present at high levels in nine of 39 specimens of infiltrating ductal carcinoma. Eight of the nine specimens of infiltrating ductal carcinoma that contained high levels of LIP were negative for estrogen receptor and progesterone receptor ($ER^-/PR^-$); all nine tumors were aneuploid and poorly differentiated, and eight of nine were highly proliferative. Of the tumors that contained LIP at low or nondetectable levels, six of 30 were $ER^-/PR^-$, 17 of 29 were aneuploid, eight of 27 were highly proliferative, and 11 of 30 were poorly differentiated.** ***Implication:*** **LIP expression should be evaluated further as a prognostic marker for patients with breast cancer.** **[J Natl Cancer Inst 1997;89:1887–91]**

Tumorigenesis often results from the failure of cells to maintain a balance between proliferation, terminal differentiation, and programmed cell death. The CCAAT/enhancer-binding protein (C/EBP) family of basic leucine zipper (bZIP), DNA-binding proteins may play a pivotal role in maintaining this balance by regulating the expression of genes involved in proliferation and terminal differentiation *(1–3)*. Currently, the genes for six C/EBPs (C/EBPα, β, δ, γ, ε, and ζ) have been characterized with the use of nomenclature introduced by Cao et al. *(3)*. With the exception of C/EBPε and ζ, all of the genes in this family are intronless and share a conserved carboxyl-terminal DNA-binding domain and bZIP dimerization domain but differ in their amino-terminal *trans*-activation domains *(4)*. The gene encoding C/EBPβ is transcribed into a single messenger RNA that is translated in the mammary gland by a leaky ribosome-scanning mechanism, resulting in the synthesis of two principal isoforms designated liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd). Consequently, studies of LAP/LIP gene regulation can be conducted only at the protein level. As with all C/EBP family members, LAP and LIP are capable of forming homodimers or heterodimers with each other as well as with other leucine zipper proteins. LIP displays an increased affinity for DNA, but it lacks a portion of its *trans*-activating domain, rendering it able to antagonize the transcriptional activation of LAP or other C/EBPs and leucine zipper proteins, in substoichiometric ratios *(5)*.

Recently, we have reported that C/EBPβ-LIP expression is elevated in transplantable and primary mouse mammary tumors of different etiologies but is not expressed in transplantable, preneoplastic mammary hyperplasias *(6)*. These data suggested that LIP expression may play a role in rodent mammary tumorigenesis, but its role in human breast cancer was undetermined. We hypothesized that an increase in the levels or activity of LIP may inhibit terminal differentiation and help facilitate uncontrolled proliferation and tumorigenesis that may result from other genetic alterations known to occur in breast cancer, e.g., amplification of growth factor receptors such as erbB2, increased expression of cyclin D1, and loss or mutation of p53. LIP is of particular interest because it represents a translationally regulated, naturally occurring, dominant-negative C/EBP family member that may play a role in breast cancer. Consequently, the purpose of this study was to determine if LIP expression is elevated in human breast cancer and whether this overexpression is associated with biologic predictors of aggressive behavior in human breast cancer, such as histologic and nuclear grade, cell proliferation, DNA ploidy, and estrogen receptor (ER) and progesterone receptor (PR) status.

## Materials and Methods

### Western Blot Analysis

Infiltrating ductal carcinoma specimens from 37 patients and normal breast tissue from 10 patients were provided by the Baylor College of Medicine Tissue Bank, Department of Pathology. Specimens of infiltrating ductal carcinoma from two patients were provided by the Providence Health Center, Waco, TX. Seven of the 10 normal tissues were paired controls from patients with infiltrating ductal carcinomas examined in this study, and the remaining three were age-matched controls. Normal tissue is defined as tissue resected greater than 2 cm from the margins of the tumor, containing minimal adipose tissue and a higher proportion of breast parenchyma. The use of discarded human tissue was approved by the Baylor Institutional Review Board. Tissues were disrupted in RIPA buffer—50 m*M* Tris–HCl (pH 7.4), 1% Nonidet P-40, 0.25% desoxycholate, 150 m*M* NaCl, 1 m*M* EGTA [i.e., ethylene glycol-bis(β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid], and 0.2% sodium dodecyl sulfate (SDS)—containing the following kinase, phosphatase, and protease inhibitors: 1 m*M* $NaVO_3$, 1 m*M* NaF, 1 m*M* $Na_2MoO_4$, 10 n*M* okadaic acid, and 1 μg/mL benzamidine, aprotinin, soybean trypsin in-

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*See* "Notes" following "References."

hibitor, and antipain. Aliquots of these lysates containing 100 μg of protein were subjected to electrophoresis on denaturing SDS–12% polyacrylamide mini gels and then transferred to polyvinylidene fluoride membranes (Millipore Corp., Bedford, MA) overnight at 75 mA. Blots were blocked 90 minutes in TBST (20 m*M* Tris [pH 7.5], 150 m*M* NaCl, and 0.5% Tween 20) containing 3% non-fat dry milk (Carnation, Glendale, CA). They were then incubated for 90 minutes in this solution containing antibodies (0.5 ng/mL) (Santa Cruz Biochemicals, Inc., Santa Cruz, CA) prepared against C/EBPβ. Blot washes consisted of TBST (without milk); the washing was done three times for 5–10 minutes each, with agitation. Blots were then incubated for 60 minutes in blocking solution containing 200 ng/mL biotinylated donkey anti-rabbit immunoglobulin (Amersham, Little Chalfont, U.K.) and then washed. Lastly, blots were incubated for 30 minutes in blocking solution containing 40 ng/mL streptavidin–horseradish peroxidase (Oncogene Science, Uniondale, NY) and washed as before. Enhanced chemiluminescence (Hyperfilm; Amersham) and chemifluorescence reagents (Storm Fluoroimager; Molecular Dynamics, Sunnyvale, CA) were used for visualization, as per the manufacturer's instructions.

To determine the sensitivity of western blot analysis for detection of C/EBPβ expression, we used both the Amersham enhanced-chemiluminescence (ECL) and the enhanced-chemifluorescence (ECF) detection systems to analyze increasing amounts of a C/EBPβ protein standard isolated from previously characterized mouse mammary tumors generated by overexpression of transforming growth factor-α (TGF-α) *(6)*. The ECL data were analyzed by use of multiple exposures of Hyperfilm and quantitated with Adobe Photoshop (Adobe Systems, Mountain View, CA), and the ECF data were analyzed by use of a STORM imaging system (Molecular Dynamics) and Image Quant software (Molecular Dynamics). Levels of LAP and LIP were linear over a larger range with the use of ECF detection (12.5–400 μg/lane) as compared with ECL detection (6.25–100 μg/lane) (data not shown). Consequently, for purposes of quantitation, each western blot was analyzed with the use of the ECF detection system, and levels of LAP and LIP were analyzed as "-fold" and/or percent change from the C/EBPβ protein standard (100 μg). Because a source of purified C/EBPβ protein was not available, an extract of TGF-α-induced mammary tumors (in RIPA buffer) that contained large amounts of the C/EBPβ isoforms was used as the standard. The values were plotted on a scatter diagram, and the data for LIP expression were separated into two distinguishable groups (data not shown). Standard values were assigned the value of 1 or 100%. High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, lower LIP (low or nondetectable) values ranged from 0.3-fold below standard values to 0. None of the data points were positioned between the values of 0.53 (53%) and 0.25 (25%). Consequently, values that were greater than 50% below the value for the C/EBPβ protein standard were classified as high, and those that were less than 25% below the standard were classified as low or nondetectable. As a visual check for uniform gel loading, all blots were stained with the Reversible Protein Detection Kit (Sigma Chemical Co., St. Louis, MO).

## Immunohistochemistry

Tissues were fixed overnight in 10% neutral buffered formalin, embedded, sectioned, deparaffinized through a graded series of xylenes and alcohols, and then rehydrated in water and phosphate-buffered saline. Antigens were retrieved by boiling the tissue sections for 10 minutes in 2 *M* urea, and endoperoxidases were blocked in a methanol solution containing 3% $H_2O_2$ for 15 minutes at room temperature. After being washed, tissue sections were incubated for 1 hour at room temperature with 10% normal goat serum (Sigma Chemical Co.) and 20% avidin blocking solution (Vector Laboratories, Inc., Burlingame, CA) in 1× PBST (i.e., phosphate-buffered saline containing 0.1% Tween 20). Excess blocking solution was drained, and polyclonal rabbit primary antibody for C/EBPβ (1:300; Santa Cruz Biochemicals, Inc.) containing 20% biotin blocking solution (Vector Laboratories, Inc.) in 10% normal goat serum was applied overnight at 4 °C. Biotinylated goat anti-rabbit secondary antibody at a 1:200 dilution in 10% normal goat serum was incubated for 30 minutes at room temperature. Tissue sections were then incubated for 30 minutes at room temperature with the preformed avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain *Elite* ABC; Vector Laboratories, Inc.). Colorimetric detection by use of the chromagen diaminobenzidine tetrahydrochloride was carried out according to the manufacturer's instructions (Vector Laboratories, Inc.). Control slides were treated identically except that 6 μg of C/EBPβ peptide (Santa Cruz Biochemicals, Inc.) was incubated with the primary antibody for 15–30 minutes at 0 °C.

## Analysis of Prognostic Indicators

Analyses of ER and PR status were performed at OncQuest, Specialty Laboratories, Inc., Santa Monica, CA, by use of a dextran–charcoal-binding assay. For consistency in histologic grading, all slides were examined by one pathologist (R. Laucirica), and the grade for each infiltrating ductal carcinoma was assessed by use of the method of Elston and Ellis *(7)*. DNA flow cytometry analysis of tumors procured at the time of surgery and MIB-1 (Immunotech, Inc., Westbrook, ME) immunohistochemistry, which detects the Ki67 nuclear antigen associated with cellular proliferation *(8)*, were conducted in the Department of Pathology, The Methodist Hospital. The DNA content (ploidy) for 37 of 39 cases was assessed by use of a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and Modfit software (Verity Software House, Topsham, ME). Nonparametric statistical analysis was performed in consultation with a biostatistician (Dr. Charles Miller, Department of Surgery, Baylor College of Medicine). *P* values were computed by Fisher's exact test (two-sided) and the Mann-Whitney *t* test (two-sided).

# Results

LIP is an amino-truncated version of the C/EBPβ LAP isoform. Therefore, C/EBPβ antibodies, which recognize a carboxy-terminal epitope in C/EBPβ proteins, detect both LAP and LIP isoforms. Accordingly, the antibodies that recognize the amino-terminal region do not detect LIP. Consequently, no antibodies that can selectively distinguish LIP are currently available. Size fractionation of the C/EBPβ protein isoforms by SDS–polyacrylamide gel electrophoresis followed by western blotting with the use of an antibody to the carboxy-terminus of C/EBPβ, however, permitted the detection of the different C/EBPβ isoforms. At present, this is the only technique available for the analysis of LIP expression.

To address the question, "Is LIP expressed in human breast cancer?," we analyzed infiltrating ductal carcinomas from 39 women, aged 26–83 years old. As evidenced by western blot analyses, levels of LAP and LIP expression within the infiltrating ductal carcinomas were quite variable among the different patients (Fig. 1, A, B, and C). Additional protein bands, representing both cross-reactive proteins and phosphorylated or other post-translationally processed LAP and LIP isoforms, were also visible in many tumors.

For the determination of the significance of elevated levels of LIP expression in some tumors, an expression level was assigned (as described in the "Materials and Methods" section) to the LIP values and was tested for association with various prognostic factors. LIP was expressed at high levels in 23% (nine of 39) of the infiltrating ductal carcinomas. Examples of high levels of LIP expression are shown in Fig. 1, B (lanes 2, 3, and 6). LIP levels designated as "high" (mean value ± 95% confidence interval: 1.59 ± 0.6) were on average 15 times greater than the levels of LIP in the lower expressing (mean value ± 95% confidence interval: 0.107 ± 0.02) or nonexpressing tumors ($P<.0001$, two-sided Mann-Whitney *t* test). In "normal" breast tissue, LIP levels were low or nondetectable above background values.

Because the amino-truncated LIP isoform has a greater DNA affinity than the LAP isoform and can heterodimerize and inhibit the transactivation ability of other C/EBPs at a substoichiometric ratio as low as 1:5 (20%), the ratio of LIP to LAP was determined for the predominant LAP isoform (35 kd) that also has the greatest transactivation potential *(5)*. In the tumors expressing the highest LIP levels, the average ratio of LIP to LAP was determined

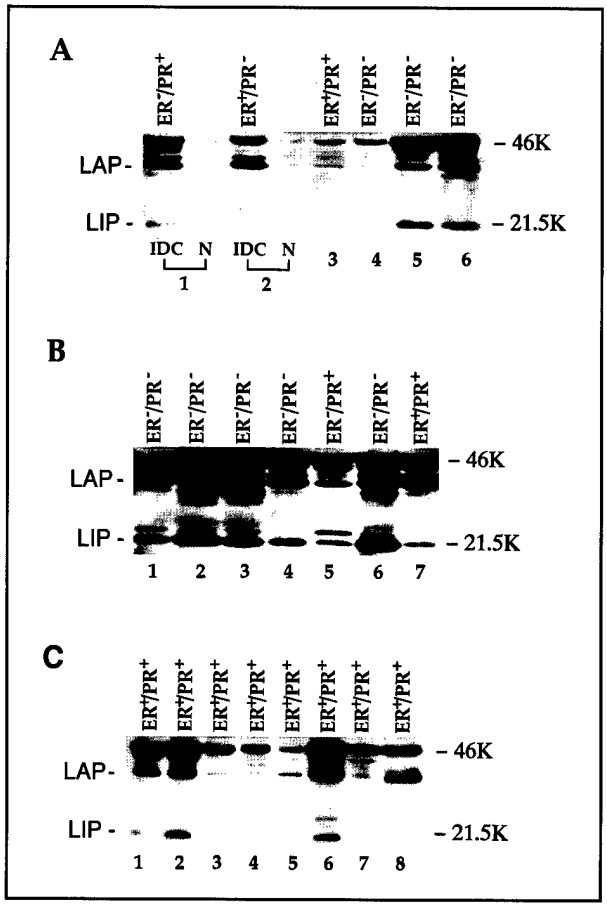


**Fig. 1.** Western blot analysis and enhanced-chemiluminescence (ECL) detection of CCAAT/enhancer-binding protein β (C/EBPβ) isoforms (liver-enriched activating protein [LAP—35 kd] and liver-enriched inhibitory protein [LIP—20 kd]) in human infiltrating ductal carcinomas. With the exception of lanes 1 and 2 in **panel A,** each lane represents the infiltrating ductal carcinoma from a different patient. Lanes 1 and 2 in **panel A** consist of an infiltrating ductal carcinoma (IDC) and its paired normal (N) tissue. One hundred micrograms of protein was analyzed per lane. The estrogen receptor (ER) and progesterone receptor (PR) status from each infiltrating ductal carcinoma is listed above the corresponding lane. The exposure time for **panel B** is not the same as that for **panel A** and **panel C,** and these western blot analyses were not all performed simultaneously. The variations in LIP levels on these ECL blots have been confirmed by use of the quantitative enhanced-chemifluorescence detection methodology described in the "Materials and Methods" section.

**Table 1.** Association of liver-enriched inhibitory protein (LIP) expression levels with steroid receptor status*

| Receptor status | LIP expression level | |
|---|---|---|
| | High | Low or nondetectable |
| $ER^-/PR^-$ | 89% (8/9) | 20% (6/30) |
| $ER^+/PR^+$ | 11% (1/9) | 60% (18/30) |
| $ER^-/PR^+$ | None | 7% (2/30) |
| $ER^+/PR^-$ | None | 13% (4/30) |

*Association of steroid receptor status with LIP overexpression in infiltrating ductal carcinomas. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) were negative for estrogen and progesterone receptor ($ER^-/PR^-$), 19 (49%) were positive for estrogen and progesterone receptor ($ER^+/PR^+$), and six (15%) were either estrogen receptor negative and progesterone receptor positive ($ER^-/PR^+$) or estrogen receptor positive and progesterone receptor negative ($ER^+/PR^-$). High LIP values ranged from 5.2-fold above the standard to 0.5-fold below the standard. Likewise, low and nondetectable LIP values ranged from 0.3-fold below standard values to 0. Frequency analysis was performed by use of two-sided Fisher's exact test ($P = .0015$).

to be 1:2.4 (42.3% ± 6% [mean ± standard error]).

Two of the most frequently used prognostic indicators in breast cancer are tumor size and lymph node status. No association was observed between these two indicators and C/EBPβ-LIP levels. Unfortunately, the tissues analyzed from the Tissue Bank of The Methodist Hospital were biased toward tumors larger than 1 cm because of the inherent difficulties in banking tissue from smaller tumors. Consequently, tissue was analyzed from tumors ranging in size from 1.2 cm to 20 cm at the largest dimension. In addition, 22 patients had metastases to the lymph node, and the lymph node status for 11 patients was not determined. The sample size for the six lymph node-negative patients and the tumors smaller than 1 cm was, therefore, not large enough to draw any statistically significant conclusions.

To determine whether an association existed between genetic instability and LIP expression in the infiltrating ductal carcinomas, we compared levels of LIP expression in 38 of 39 tumor specimens with DNA ploidy. Nine (100%) of the nine tumors that expressed LIP at high levels were aneuploid. The tumors that expressed LIP at either low or nondetectable levels were more evenly distributed: 17 (59%) of 29 were aneuploid, whereas 12 (41%) of 29 were diploid ($P = .0356$, two-sided Fisher's exact test).

Because loss of ER expression is often associated with a poor clinical outcome *(9),* we next determined whether steroid receptor status in these tumors was associated with LIP expression. Of the 39 infiltrating ductal carcinomas examined, 14 (36%) of 39 specimens were negative for ER and PR ($ER^-/PR^-$), and 19 (49%) of 39 specimens were positive for ER and PR ($ER^+/PR^+$). Eight (89%) of nine $ER^-/PR^-$ tumors expressed high levels of LIP, whereas only one (11%) of nine $ER^+/PR^+$ tumors exhibited high levels of LIP expression ($P = .0015$, two-sided Fisher's exact test) (Table 1).

Each tumor was graded blindly by use of the system of Elston and Ellis *(7),* which examines and scores the degree of tubule formation, the degree of nuclear pleomorphism, and the number of mitotic counts. All of the high LIP-expressing tumors (nine of nine) were classified as poorly differentiated (i.e., Elston–Ellis grade III) and highly proliferative tumors. In contrast, all three histologic grades (grade III—36.7% [11 of 30]; grade II—30% [nine of 30]; and grade I—33.3% [10 of 30]) were evenly distributed between the tumors expressing LIP at low or nondetectable levels ($P = .013$, two-sided Fisher's exact test). The proliferative fraction (determined by either MIB-1 immunohistochemistry and/or by DNA flow cytometry) also displayed an association with LIP expression. In the infiltrating ductal carcinomas that contained high LIP levels, eight (89%) of nine specimens contained a high fraction of proliferative cells, but only eight (30%) of 27 of the low or nondetectable LIP-expressing tumors contained a high fraction of proliferative cells ($P = .0046$, two-sided Fisher's exact test).

Although it was not possible to localize LIP expression to specific cell types, immunocytochemical staining was performed on a limited number of the infiltrating ductal carcinomas and their surrounding tissue to determine which cells expressed the C/EBPβ isoforms. These results were consistent with those obtained from the previous western blot analyses and revealed strong C/EBPβ expression in pleomorphic nuclei of grade III, $ER^-/PR^-$ tumors (Fig. 2, A) and weaker C/EBPβ expression in the nuclei of grade I, $ER^+/PR^+$ tumors (Fig. 2,

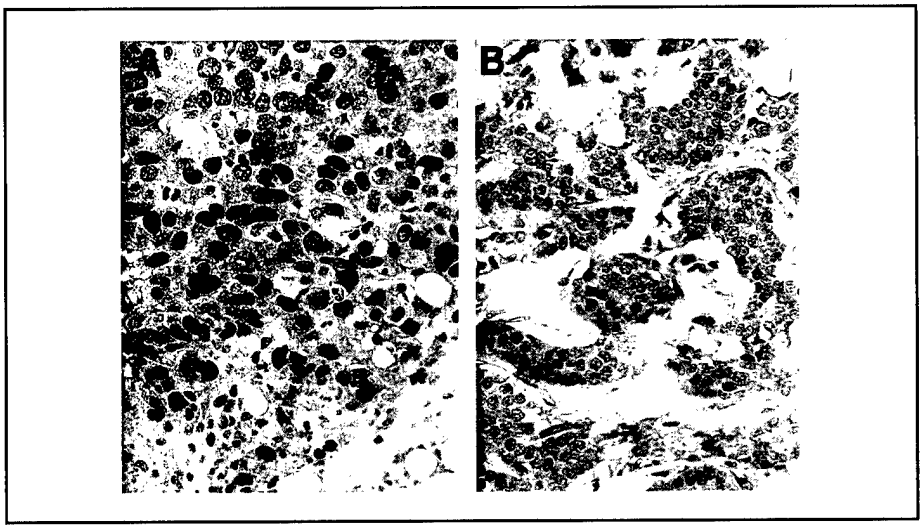


**Fig. 2.** Immunohistochemical localization of CCAAT/enhancer-binding protein β (C/EBPβ) isoform expression in a human Elston–Ellis grade III, estrogen receptor-negative and progesterone receptor-negative, infiltrating ductal carcinoma **(A)** and an Elston–Ellis grade I, estrogen receptor-positive and progesterone receptor-positive, infiltrating ductal carcinoma **(B).** Note the intense staining of pleomorphic nuclei in **panel A** and much weaker staining in **panel B.** In agreement with these results, western blot analysis of the infiltrating ductal carcinoma in **panel A** shows strong expression of the C/EBPβ isoforms (*see* Fig. 1, B, lane 6), but the infiltrating ductal carcinoma in **panel B** expresses the isoforms at a much lower level (*see* Fig. 1, A, lane 3). Viewed with a 40× objective.

B). Surrounding normal tissue also expressed C/EBPβ in both the epithelium and stroma (data not shown). The specificity of the staining was confirmed by selective competition with the peptide against which the carboxy-terminal antiserum was generated, but not by a nonspecific peptide (data not shown). These results from immunohistochemical analyses have confirmed that the C/EBPβ isoforms are expressed strongly in malignant epithelial cells of aggressive, poorly differentiated infiltrating ductal carcinomas (Fig. 2, A and B). However, development of a double fluorescent-imaging technique using amino- and carboxy-terminal antisera will be required to detect the selective localization of LIP expression in the future. Examination of hematoxylin–eosin-stained microscopic tissue sections of both the high and the low LIP-expressing tumors also demonstrated that many of the less aggressive, low LIP-expressing tumors possessed large sheets of tumor epithelium, as illustrated in Fig. 2. Consequently, differences in the ratio of LIP to LAP cannot be accounted for by variations in epithelial cell number.

## Discussion

Breast cancer is thought to progress through multiple morphologic stages, beginning with typical hyperplasia and progressing through atypical hyperplasia, *in situ* carcinoma, invasive carcinoma, and eventual metastasis. Invasive carcinomas are also heterogeneous with respect to their proliferative and invasive potential. In this study, C/EBPβ protein isoforms and, in particular, the naturally occurring, dominant-negative LIP isoform have been detected and are more highly expressed in $ER^-/PR^-$, aneuploid, Elston–Ellis grade III infiltrating ductal carcinomas than in the less aggressive, $ER^+/PR^+$, diploid, Elston–Ellis grade I tumors. This observation is consistent with the hypothesis that the C/EBPβ isoforms may play a role in regulating terminal differentiation and cell cycle progression, as illustrated in Fig. 3.

Proliferative diseases such as cancer often result from failure to withdraw from the cell cycle at the $G_1$ checkpoint. Multiple signal transduction pathways, generated by diverse extracellular and intracellular factors, converge at this restriction point and influence cell cycle progression. This advancement beyond late $G_1$ is believed to be a result of the phosphorylation and consequent inactivation of the retinoblastoma protein (Rb). It has been demonstrated that Rb interacts directly with and activates all of the C/EBPβ isoforms *(10);* however, how this interaction

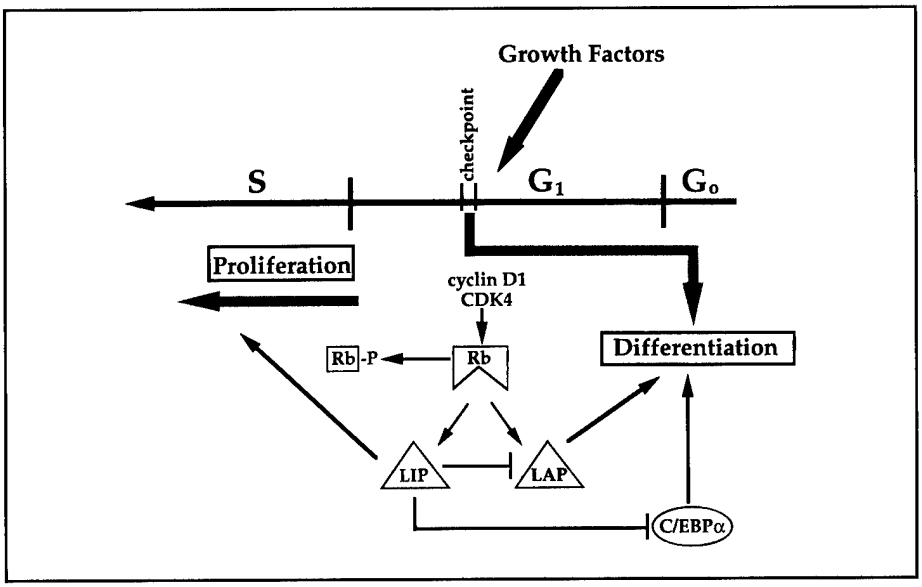


**Fig. 3.** Hypothetical model for the role of CCAAT/enhancer-binding proteins (C/EBPs) in cell cycle progression. The interaction of retinoblastoma protein (Rb) with the C/EBPβ–liver-enriched activating protein (LAP—35 kd) and liver-enriched inhibitory protein (LIP—20 kd) isoforms may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland (*see* text for details). Studies in adipocytes have demonstrated that C/EBPβ and C/EBPδ are involved in early proliferative and differentiative processes, leading to the activation of C/EBPα, which then contributes to terminal differentiation by arresting adipocyte proliferation *(16,17).* In HepG2 hepatoma cells, C/EBPβ-LAP has been reported to inhibit cell cycle progression before the $G_1$/S boundary, and this effect can be antagonized by expression of the dominant-negative LIP isoform, thereby promoting cell proliferation *(11).* In the rat mammary gland, C/EBPα expression is highest at lactation, when mammary epithelial cells undergo terminal differentiation, and C/EBPβ expression with an elevated ratio of LIP to LAP is highest during pregnancy, a period of lobuloalveolar proliferation *(12).* The ratio of LIP to LAP decreases almost 100-fold at the onset of lactation.

affects Rb activity is not known. This interaction may provide a novel mechanism to regulate the switch between terminal differentiation and proliferation in the mammary gland and supports the hypothesis that the ratio of C/EBPβ isoforms may play a role in the control of cell cycle progression. We, therefore, propose that increased LIP expression may inhibit terminal differentiation and provide a selective growth advantage facilitating tumor progression (Fig. 3). Support for this hypothesis comes from the observations that the ratio of LIP to LAP is regulated during proliferative phases of both liver and mammary gland development *(5,11,12)*.

One caveat that must be considered in the interpretation of the foregoing results is the cellular heterogeneity that exists between breast tumors of the same type. For example, when infiltrating ductal carcinomas are compared, ER and PR levels may vary considerably in cells within a given tumor *(13,14)*. The degree of desmoplasia, lymphovascular invasion, and necrosis can also vary from patient to patient. Homogenization of tumor specimens for western blot or steroid receptor analyses can result in a misleading "dilutional" effect and does not permit direct cellular comparisons. This heterogeneity may be partly responsible for the variations observed in the LAP and LIP levels between infiltrating ductal carcinomas and tempers the associations of LIP expression levels with steroid receptor status and DNA ploidy. Furthermore, these studies do not permit an evaluation of ratios of LIP to LAP within individual cells of differing proliferative potentials within these tumors.

Mammary tumorigenesis is thought to result from multiple molecular changes, such as the activation of oncogenes by mutation, the overexpression of growth factors and their receptors, and/or the inactivation of tumor suppressor genes. It has been proposed that multiple molecular changes lead to a malignant phenotype, whereas, fewer "hits" are necessary for a benign tumor *(15)*. Our study suggests that overexpression of the naturally occurring, dominant-negative transcription factor C/EBPβ-LIP may be one of the molecular events that predisposes breast cells to a selective growth advantage, resulting in progression of infiltrating ductal carcinomas; however, additional tumors need to be examined to confirm our hypothesis.

These studies do not answer the question of whether overexpression of LIP, in the most advanced, aggressive tumors, facilitates tumor progression or if overexpression is simply a result of increased proliferation. These type of functional studies cannot be performed with patient biopsy specimens but require the use of animal or cell culture models. In this regard, we have recently generated transgenic mice that selectively express high levels of LIP in cells of the mammary gland (Zahnow CA, Rosen JM: unpublished observations). Studies are also in progress to determine the effects of regulatable LIP expression on cell cycle progression in mammary epithelial cells. These types of experiments will be required to determine if LIP expression is a cause or an effect of mammary tumorigenesis. Notwithstanding this determination, LIP expression may provide a useful additional marker for the identification of breast tumors in patients with a poor clinical outcome.

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## Notes


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