C/EBPβ-mediated transcriptional regulation of *bcl-xl* gene expression in human breast epithelial cells in response to cigarette smoke condensate

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Abstract

In previous studies, we have shown that cigarette smoke condensate (CSC), a surrogate for cigarette smoke, is capable of transforming the spontaneously immortalized human breast epithelial cell line, MCF10A. These transformed cells displayed upregulation of the anti-apoptotic gene, *bcl-xl*. Upregulation of this gene may impede the apoptotic pathway and allow the accumulation of DNA damage that can lead to cell transformation and carcinogenesis. In the present study, we have determined the mechanism of CSC-mediated transcriptional upregulation of *bcl-xl* gene expression in MCF10A cells. We cloned the human *bcl-xl* promoter (pBcl-xLP) and identified putative transcription factor binding sites. Sequential deletion constructs that removed the putative *cis*-elements were constructed and transfected into MCF10A cells to determine the CSC-responsive *cis*-element(s) on the pBcl-xLP. Gel-shift, supershift, and chromatin immunoprecipitation (ChIP) analysis confirmed that C/EBPβ specifically bound to a C/EBP-binding site on the pBcl-xLP *in vitro* and *in vivo*. Additionally, overexpression of C/EBPβ-LAP2 stimulated pBcl-xLP activity and Bcl-xL protein levels, which mimicked the conditions of CSC treatment. Our results indicate that C/EBPβ regulates *bcl-xl* gene expression in MCF10A cells in response to CSC treatment, therefore making it a potential target for chemotherapeutic intervention of cigarette smoke-induced breast carcinogenesis.

Keywords

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Introduction

Environmental factors, such as cigarette smoking, have long been suspected to contribute to human breast cancer. Epidemiological studies have reported conflicting associations between cigarette smoking and breast cancer risk. While some studies have failed to show increased breast cancer in women who smoke (Fink and Lash, 2003; Lash and Aschengrau, 2002) other studies indicate a role of cigarette smoke in the etiology of breast cancer (Bennett *et al.*, 1999; Wells, 2000). Some recent studies have reported that smoking increases breast cancer, particularly in those females that start smoking at younger ages (Johnson, 2005; Nagata *et al.*, 2006; Reynolds *et al.*, 2004).

Biological studies support the hypothesis that cigarette smoking plays a role in breast carcinogenesis. Chemical and animal bioassay studies indicate that tobacco smoke contains about 4,800 compounds and approximately 100 of these are carcinogens, co-carcinogens or mutagens (Hecht, 2002; Hoffmann *et al.*, 2001). Tobacco smoke compounds such as benzo[α]pyrene (B[α]P) (el-Bayoumy *et al.*, 1995) and 7, 12-dimethylbenzanthracene (DMBA) (Kumar *et al.*, 1990) are proven mammary carcinogens. Evidence suggests that smokers metabolize cigarette constituents in their breast tissue; nicotine and its metabolite, cotinine, have been found in the breast secretions of non-lactating, women smokers (Petrakis *et al.*, 1978) and DNA adducts have also been reported in the breast tissue of smokers (Perera *et al.*, 1995). Cigarette smoke condensate (CSC) has been used as a surrogate to study the genotoxic and other effects of cigarette smoke in many model systems (DeMarini, 2004). Previously, we have shown that the spontaneously immortalized human breast epithelial cell line, MCF10A, is transformed by CSC in culture (Narayan *et al.*, 2004). MCF10A cells spontaneously arose from culturing MCF10M cells, which were derived from a 36-year old parous pre-menopausal woman with extensive fibrocystic disease, but no family history or histological evidence of breast malignancy. MCF10A cells are estrogen receptor negative (ER-) and have characteristics of normal cells including: growth factor-dependency, anchorage-dependent growth, and lack of tumorigenecity in nude mice. Therefore, these cells are considered a model for normal breast epithelial cells and provide the opportunity to study the mechanism of transformation and tumorigenesis (Soule *et al.*, 1990; Tait *et al.*, 1990). After a single CSC treatment, MCF10A cells displayed transformed characteristics such as increased proliferation, and anchorage-independent growth and also showed increased expression of *bcl-xl* mRNA and protein levels (Narayan *et al.*, 2004).

Bcl-xL is an anti-apoptotic member of the Bcl-2 protein family most closely related to the founding member anti-apoptotic Bcl-2 (Boise *et al.*, 1993; Grillot *et al.*, 1997). *Bcl-xl* is upregulated in breast cancer cell lines and primary breast tumors, and is considered as a marker for increased tumor grade and nodal metastasis (Olopade *et al.*, 1997; Schott *et al.*, 1995). The evasion of apoptosis is considered one of the six hallmarks of cancer (Hanahan and Weinberg, 2000). Cells expressing Bcl-xL are more likely to survive following DNA damage and can potentially accumulate new somatic mutations at higher frequencies (Cherbonnel-Lasserre *et al.*, 1996). The resulting persistent accumulation of damage and mutations could lead to cell cycle deregulation, transformation, and eventually carcinogenesis. We hypothesized that CSC treatment caused increased expression of Bcl-xL leading to transformation of MCF10A cells. In the present study, we determined the

mechanism by which CSC causes transcriptional upregulation of *bcl-xl* gene expression in MCF10A cells. Our results suggest, for the first time to our knowledge, that C/EBPβ binds the *bcl-xl* promoter and induces its activity in MCF10A cells in response CSC treatment.

Results

CSC treatment induces bcl-xl mRNA and protein levels in MCF10A cells

To determine the effect of CSC treatment on *bcl-xl* gene levels, we treated MCF10A cells with increasing amounts of CSC for 24 h and *bcl-xl* mRNA levels were measured by RT-PCR (Fig. 1A). In these cells after normalization with *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* mRNA, the *bcl-xl* mRNA expression slightly decreases at lower concentrations (2.5 μg/mL) and increases at higher concentrations of CSC treatment as compared to untreated cells. To confirm the subsequent induction of Bcl-xL protein levels, cells were treated with 25 μg/mL of CSC for different time intervals and with increasing concentrations of CSC for 24 h. Western analysis was used to determine the protein levels. The results showed an increased level of Bcl-xL protein in both time- (Fig. 1B, upper panel) and concentration-dependent manners (Fig. 1B, lower panel). These results indicate that CSC treatment induces *bcl-xl* mRNA and protein levels in MCF10A cells. The concentration of CSC used in these experiments was in the range of 0–50 μg/ml. This concentration of CSC is not as high as compared to the exposure with 10 mg of tar (CSC) present in each cigarette, which contains about 6.4 ng of benz(*a*)pyrene, a well-known mammary carcinogen (Stabbert *et al.*, 2003). In human body, components of cigarette smoke can be transported to distal organs by blood including breast. Although a critical level of carcinogens is required to trigger the process of DNA damage and carcinogenesis, it can be an accumulative effect of cigarette smoke carcinogens, instead of a single event leading to carcinogenesis in human body.

CSC induces bcl-xl promoter activity in MCF10A cells

To determine how *bcl-xl* was induced by CSC, the human *bcl-xl* promoter (Grillot *et al.*, 1997; Sevilla *et al.*, 1999) was cloned into a pGL3-Basic Luciferase Vector (Promega Corp, Madison, WI) as described in Methods and materials and was named pBcl-xLP. Transcription initiation sites were located at +1 and +78 and putative *cis*-regulatory elements on the pBcl-xLP were identified (Fig. 2; Grillot *et al.*, 1997; Sevilla *et al.*, 1999). The pBcl-xLP promoter had consensus sites for several transcription factors that were reported in earlier studies, such as NF-κB, Oct1, Sp proteins, GATA, STAT, and others. To determine whether the pBcl-xLP was responsive to CSC treatment, promoter constructs were transfected into MCF10A cells and then the cells were treated with 25 μg/mL CSC for the time course (0 to 48 h) or treated with varying concentrations (0–50 μg/mL) of CSC for 24 h. Promoter activity was measured with luciferase assays. The promter construct pBcl-xLP(−145,+707) was not induced by CSC treatment (data not shown); however, the promoter construct pBcl-xLP(−54,+647) was found to have basal promoter activity. We considered this as a wild-type promoter and will be referred to as pBcl-xLP from here onward. CSC treatment of the cells induced pBcl-xLP promoter activity in a time-dependent manner, with the highest time point of induction at 24 h (Fig. 3A) and in a concentration-dependent manner, with the highest induction at 50 μg/mL of CSC treatment (Fig. 3B).

Based upon these experiments, the optimum conditions were determined to be 25 μg/mL concentration of CSC and 24 h of treatment time. These studies suggest that pBcl-xLP promoter activity was induced in MCF10A cells by CSC treatment in a time- and concentration-dependent manner.

C/EBP-binding sites on the pBcl-xLP are responsive to CSC treatment

Putative *cis*-elements on the *bcl-xl* promoter represent possible binding sites for transcription factors that can activate or repress the transcription of this gene. To determine which transcription factor was responsive for increasing pBcl-xLP expression in treated cells, sequential deletion constructs (Fig. 4A) were transfected into MCF10A cells and treated with 25 μg/mL of CSC for 24 h, and promoter activity was measured. The results indicated that pBcl-xLP(−54,+647) activity was significantly induced by CSC (Fig. 4B). Basal promoter activity was reduced with pBcl-xLP(−28,+707), which reflected the loss of the C/EBP-binding site-I, but the CSC response was maintained, suggesting that C/EBP-binding site-I was important for the basal *bcl-xl* promoter activity and that it may or may not have been responsive to CSC treatment. The *bcl-xl* promoter activity continued to decrease as other elements were deleted. However, the CSC response was maintained up to pBcl-xLP(−28,+222). The promoter activity decreased at the next construct, pBcl-xLP(−28,+132), which represented the loss of the C/EBP-binding site-II. The loss of this site resulted in an unrecoverable decrease in promoter activity.

To further examine whether C/EBP-binding site-I and II were necessary for CSC-induced promoter activity, we mutated these sites of the pBcl-xLP promoter by site-directed mutagenesis. The mutant constructs were then transfected into MCF10A cells that were subsequently treated with CSC. While the wild-type promoter showed a significant induction of activity in response to CSC treatment, neither mutant promoter constructs showed a significant change in response to treatment (Fig. 5A). These results indicate that C/EBP-binding site-I and II might be CSC-responsive element on the pBcl-xLP in MCF10A cells.

C/EBPβ binds to the pBcl-xLP in MCF10A cells in vitro and in vivo

Two putative C/EBP sites on the pBcl-xLP were identified as being inducible by CSC. C/EBPβ is one of the six C/EBP proteins and evidence suggests that it has role in human breast carcinogenesis; however, this role is not completely understood (Milde-Langosch *et al.*, 2003;Zahnow, 2002). Its putative role in human breast carcinogenesis made C/EBPβ an appropriate target protein responsible for the upregulation in *bcl-xl* in CSC-treated MCF10A cells. Western analysis was used to confirm that C/EBPβ was induced by CSC treatment. Two C/EBPβ isoforms LAP1 (45-kDa) and LAP2 (42-kDa) were detected in whole cell extracts from CSC-treated MCF10A cells; however, LIP (20-kDa) was poorly expressing and could not be detected in these cells (Fig. 5B and C). Only LAP2 levels were significantly increased in a time- and concentration-dependent manner (Fig. 5B and C, respectively). These experiments suggested that C/EBPβ protein levels are induced by CSC treatment.

Then we used electrophoretic mobility-shift assay (EMSA) to characterize the C/EBP-binding proteins binding to the pBcl-xLP promoter in response to CSC treatment. For these experiments, ^{32}P-labelled double-stranded probes, identical to C/EBP-binding site-I and site-II sequences were incubated with MCF10A nuclear extract. Two DNA-protein complexes (shifted bands-I and -II) were visualized with autoradiography (Fig. 6A). These bands represented nuclear proteins binding to the C/EBP regulatory elements on the *bcl-xl* promoter. To further determine the binding specificity of nuclear proteins with C/EBP-binding site-I and II oligonucleotides, the competition experiments were performed with molar excess of appropriate unlabelled wild-type or mutant oligonucleotides (Fig. 6A). For C/EBP-binding site-I and II, the unlabelled wild-type oligonucleotide competed out the ^{32}P-labelled probe, in a concentration-dependent manner, as expected. However, the unlabelled C/EBP site-I mutant oligonucleotide also competed out the ^{32}P-labelled probe. This indicated one of the two scenarios: 1) the mutant is not sufficient enough to decrease binding, or 2) the binding at this site is non-specific. Further specificity was tested by adding increasing concentrations of a nonspecific, unlabelled GAGA probe to the reactions as indicated. The unlabelled probe also competed with the ^{32}P-labelled wild-type probe for C/EBP-binding site-I, confirming that binding to C/EBP site-I was non-specific. Conversely, the binding at C/EBP-binding site-II was not competed out by either the unlabelled mutant or the unlabelled nonspecific GAGA oligonucleotide, suggesting that MCF10A nuclear extract contained a protein that specially bound to C/EBP-binding site-II.

The protein binding to C/EBP-binding site-II was further confirmed with super-shift analysis. The three C/EBP proteins (C/EBPα, β, and δ) are expressed in mammary tissue (Gigliotti *et al.*, 2003; Sabatakos *et al.*, 1998). To determine whether the binding was specific to either protein, antibodies specific to each were added to the reaction mixtures. No super-shifted bands are observed in reactions with the ^{32}P-C/EBP-binding site-I. ^{32}P-C/EBP-binding site-II reactions showed a super-shifted band only with the addition of anti-C/EBPβ antibody (Fig. 6B, compare lane 5 with 6).

The effects of CSC treatment on super-shift analysis were also analyzed at C/EBP site-II. Reactions containing nuclear extract from either control or CSC-treated MCF10A cells were assembled. With control nuclear extract, addition of anti-C/EBPβ antibody resulted in a super-shift similar to the one in Figure 6B (Fig. 6C, compare lane 1 with 2). CSC treatment shifted band-I and showed an increased binding at shifted band-II, indicating an increased level of C/EBP-binding proteins after CSC treatment. The shifted band-I with the nuclear extract from CSC-treated cells as compared to the control cells could have been due to post-translational modification in the C/EBP-binding proteins. The addition of anti-C/EBPβ antibody caused an additional super-shift of band-I with nuclear extract of control cells and showed a little super-shift with the nuclear extract of CSC-treated cells (Fig. 6C, compare lane 1 with 2 and 3 with 4, respectively). There was no super-shift with anti-C/EBPβ antibody of band-II with the nuclear extract of CSC-treated cells. These experiments confirmed that C/EBPβ specifically binds only to the C/EBP-binding site-II on the pBcl-xLP and its binding is increased by CSC treatment, indicating that the C/EBP site-II is the target of CSC-mediated upregulation of *bcl-xl* gene expression.

To confirm that C/EBPβ binds the *bcl-xl* promoter *in vivo*, MCF10A cells were treated with increasing concentrations of CSC for 24 h and ChIP analysis was performed as described in Material and Methods. PCR of the resulting DNA was performed with primers specific to the pBcl-xLP C/EBP-binding site-II. The control cells had an initial binding of C/EBPβ to the *bcl-xl* promoter. This binding slightly decreased at 10 μg/mL of CSC and then increased at 25 and 50 μg/mL concentrations of CSC treatment (Fig. 7A). These results showed a similar pattern to that observed in the RT-PCR of *bcl-xl* in Figure 1A, indicating that *bcl-xl* mRNA levels corresponded with C/EBPβ-binding to and regulating the *bcl-xl* promoter activity in CSC-treated MC10A cells.

C/EBPβ overexpression mimics pBcl-xLP promoter activity and protein expression of CSC treatment in MCF10A cells

To demonstrate that CSC treatment increases C/EBPβ levels, which in turn, binds and regulates *bcl-xl* promoter activity, we recapitulated this condition by overexpressing C/EBPβ proteins and measuring pBcl-xL promoter activity in MCF10A cells. Each C/EBPβ construct induced promoter activity; however, only hLAP2 had a significant induction when compared to the empty pCDNA3.1 vector (Fig. 7B). To determine the effect on Bcl-xL protein levels, each construct was separately transfected into MC10A cells. After the overexpression, cells were processed for Western analysis of C/EBPβ isoforms and Bcl-xL protein levels. A very efficient overexpression of C/EBPβ isoforms, hLAP1, hLAP2 and hLIP, were observed in MCF10A cells (Fig. 7C). The Bcl-xL protein levels were similar in control cells and cells transfected with the empty pCDNA3.1 vector. The overexpression of hLAP1 slightly increased Bcl-xL protein levels, while LAP2 showed the most significant increase of Bcl-xL. Conversely, hLIP expression caused a decrease in Bcl-xL protein (Fig. 7C). LIP acts as a transcriptional dominant negative factor of hLAP1 and hLAP2 isoforms (Descombes and Schibler, 1991). In our experiments, results showed its dominant negative effect as the overexpression of hLIP decreases the expression of hLAP1 and hLAP2 in MCF10A cells (Fig. 7C; Bundy et al., 2005).

Next, we determined whether the C/EBP-binding site-I and/or II are responsive to the overexpression of C/EBPβ isoforms. We co-transfected C/EBP-binding site-I or II mutant pBcl-xL plasmid with one of the C/EBPβ isoforms overexpressiong plasmids. The results showed that the overexpression of C/EBPβ isoforms with pBcl-xLP mutants disrupted the promoter activity (Fig. 8A and B). Although mutations in both C/EBP-binding site-I and II abolished the hLAP2- mediated pBcl-xLP promoter activity, the effect was more prominent at mutations on C/EBP-binding site-II, which is consistent with gel-shift, ChIP analysis, and overexpression results. The decreased activity of C/EBP-binding site-I mutant pBcl-xLP promoter by hLIP overexpression could be due to its dominant negative effect with hLAP2 on C/EBP-binding site-II (Fig. 8A). The effect of hLIP overexpression becomes less prominent when used with C/EBP-binding site-II mutant pBcl-xLP promoter (Fig. 8B). Thus, the presence of C/EBP-binding site-II was important for the CSC-induced and C/EBPβ-induced upregulation of *bcl-xl* promoter activity in MCF10A cells. Taken together these data indicated that C/EBPβ, especially LAP2 was necessary for the upregulation of *bcl-xl* and mimics CSC treatment in MCF10A cells.

Discussion

In a previous study, CSC-induced transformation of MCF10A cells has been described (Narayan *et al.*, 2004). In this study, the MCF10A cell line was utilized to determine the mechanism by which cigarette smoke exposure might be linked to transformation and breast carcinogenesis. Among several aspects that might play a role in CSC-induced transformation of normal breast epithelial cells such as compromised DNA repair (Kundu *et al.*, 2007), increased expression of neuropilin-1 (Narayan *et al.*, 2004), and decreased expression of S100A2 (Feng *et al.*, 2001), we focused on the mechanism of transcriptional regulation of anti-apoptotic gene, *bcl-xl*. The *bcl-xl* mRNA and protein levels were increased in MCF10A cells after CSC treatment, which was consistent with our previous findings (Narayan *et al.*, 2004). The increase in *bcl-xl* mRNA occurred through a biphasic mechanism. The base-line levels of *bcl-xl* in untreated cells ensured the survival of the cells in culture. CSC causes DNA damage in MCF10A cells (Kundu *et al.*, 2007) and they respond by triggering DNA repair pathways (Narayan *et al.*, 2004). DNA damage overloads the repair mechanisms and most cells underwent apoptosis, resulting in the decrease in *bcl-xl* mRNA levels after the first treatment. The surviving cells were responsible for the remaining low levels of *bcl-xl* expression after the initial treatment. Cells expressing higher levels of *bcl-xl* were not removed by apoptosis and subsequent treatments produced more DNA damage, persistent mutations, and the interruption of important cell regulatory mechanisms. As a result, *bcl-xl* expression continued to increase in a concentration-dependent manner.

Then, we determined whether the increased *bcl-xl* mRNA level by CSC treatment was at the transcriptional level. To determine CSC-induced transcriptional mechanism, we used a *luciferase*-reporter assay system for these studies. Human *bcl-xl* gene's promoter cloned into the *luciferase*-reporter gene (pBcl-xLP) was responsive to CSC treatment when transfected into MCF10A cells. Promoter deletion studies suggested that two C/EBP *cis*-elements were responsible for the most significant CSC-induced increase in promoter activity. Site-directed mutagenesis confirmed that CSC-induced pBcl-xLP activity was attenuated in the absence of both C/EBP-binding sites, and the protein levels of C/EBPβ were induced by CSC treatment. However, EMSA confirmed that C/EBPβ bound only to the *bcl-xl* promoter at C/EBP-binding site-II and CSC treatment increased C/EBPβ-binding only to C/EBP-binding site-II *in vitro* and *in vivo*. This result is not surprising because C/EBP-binding site-I has parts of the consensus sequence, while C/EBP-binding site-II, contains 100% of the core C/EBP consensus sequence. Overexpression studies also confirmed that the ectopic expression of C/EBPβ induced pBcl-xLP promoter and Bcl-xL proteins levels. Site-directed mutagenesis showed that C/EBP-binding sites on the pBcl-xLP were necessary for C/EBPβ to properly regulate pBcl-xLP activity.

C/EBPβ expression is a critical component in the control of mammary epithelial cell proliferation and differentiation in the functional mammary gland (Robinson *et al.*, 1998; Seagroves *et al.*, 1998). C/EBPβ is increased in human breast cancer (Eaton *et al.*, 2001; Milde-Langosch *et al.*, 2003); however, the mechanism(s) by which it influences breast carcinogenesis is not well established. In the present study, only C/EBPβ-LAP2 protein levels were induced in time- and concentration-dependent manner following CSC treatment.

Additionally, only LAP2 significantly induced pBcl-xLP activity and Bcl-xL protein levels. Although C/EBPβ-LAP2 has not been fully implicated in human breast carcinogenesis, studies support its role in the disease. LAP2 is the most prevalent form of C/EBPβ in human breast cancer cells (Eaton *et al.*, 2001) and the overexpression of C/EBPβ-LAP2 has been implicated in the transformation of human breast epithelial cells. MCF10A cells infected with a LAP2-expressing virus became anchorage independent, expressed markers of epithelial-mesenchymal transition (EMT), acquired invasive phenotypes (Bundy and Sealy, 2003), gained epidermal growth factor (EGF)-independent growth, and had disruption of normal acinar structure when grown on basement membrane (Bundy *et al.*, 2005). These studies suggest that aberrant expression of C/EBPβ isoforms, especially LAP2, contributes to breast carcinogenesis.

The following model is proposed as a starting point to uncovering the role of C/EBPβ in the upregulation of *bcl-xl* in MCF10A cells treated with CSC (Fig. 8C). When human breast epithelial cells are exposed to CSC, cells are damaged and most undergo cell cycle arrest and/or apoptosis (Narayan *et al.*, 2004). In the few surviving cells, C/EBPβ protein is activated by an unknown mechanism, triggering the dimerization of two C/EBPβ-LAP2 monomers. These homodimers bind to C/EBP site-II on the *bcl-xl* promoter and transcriptionally activate the mRNA and subsequent protein expression levels of Bcl-xL. Increased levels of Bcl-xL impede the apoptotic pathway, allowing for the accumulation of DNA damage (Mendez *et al.*, 2005; Mendez *et al.*, 2001). When genes involved in DNA repair or the apoptotic pathway are also altered, the accumulation of DNA damage can lead to cell cycle deregulation. Disruption of apoptotic pathways, may also allow for damaged cells to survive and acquire the characteristics of transformed cells. Cells overexpressing Bcl-xL can adapt to new microenvironments (Espana *et al.*, 2004; Fernandez *et al.*, 2002; Mendez *et al.*, 2006; Rubio *et al.*, 2001), have increased potential to metastasize (Fernandez *et al.*, 2002; Rubio *et al.*, 2001) and are also more prone to be resistant to chemotherapy and radiation therapy (Cherbonnel-Lasserre *et al.*, 1996; Datta *et al.*, 1995; Fernandez *et al.*, 2002; Simonian *et al.*, 1997). All of these factors contribute to the initiation and promotion of breast carcinogenesis.

The results of this study indicate that C/EBPβ, especially LAP2, is at least one of the transcription factors that regulate the induction of *bcl-xl* mRNA and protein levels in MCF10A cells treated with CSC. This and other studies place C/EBPβ as a potential oncogene in breast cancer. The current study not only provides insight to the mechanism of cigarette smoke-induced breast epithelial cell transformation and carcinogenesis, it adds to the literature that supports the link between cigarette smoking and increased breast cancer risk.

Materials and Methods

Preparation of CSC

CSC was prepared from the University of Kentucky Reference Cigarette 1R4F (Davis, 1984; Sullivan, 1984) as described earlier (Hsu *et al.*, 1991).

Culturing of MCF10A Cells

MCF10A cells were cultured and maintained in DMEM/F12 (50:50 *v/v*) Mixture with L-glutamine and 15 mM Hepes (Mediatech, Manassas, VA). Medium was supplemented with 5% horse serum, 100U/mL penicillin/streptomycin, 0.5 μg/mL hydrocortisone, 100 ng/mL cholera toxin, 10 μg/mL insulin, and 10 ng/mL epidermal growth factor. The cells were grown in a humidified atmosphere of 5% CO_2 at 37°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA was isolated with TRIzol reagent (Invitrogen Corp., Carlsbad, CA). For RT-PCR, a total of 0.5 μg RNA was used to make cDNA as per manufacturer instructions (Invitrogen Corp., Carlsbad, CA). Two micrograms of synthesized cDNA was used for PCR amplification with primers specific for *bcl-xl* (800 bp) or GAPDH (320 bp). The primers used were Bcl-xL sense: 5'-TTGGACAATGGACTGGTTGA-3', anti-sense: 5'-GTAGAGTGGATGGTCAGTG-3' and GAPDH sense: 5'-GGGAAGCCACTGGCATGGCCTTCC-3', anti-sense: 5'-CATGTGGGCCATGAGGTCCACCAC-3'. The conditions for PCR amplifications were: 1 cycle of 94°C for 2 min; 35 cycles of 94°C for 20 sec, 58°C for 30 sec, 72°C for 1 min.

Western Blot Analysis

MCF10A cells were plated on 150 mm plates and allowed to grow to 50–60% confluency and then treated with CSC as described in the figure legends. After the treatment cells were harvested and processed for the preparation of whole cell extract. The protein levels were determined by Western blot analysis (Narayan and Jaiswal, 1997). The antibodies anti-Bcl-xL, anti-C/EBPβ, and anti-β-actin were purchased from Santa Cruz Biotechnology, CA.

Cloning of the Human bcl-xl Promoter (pBcl-xLP)

The human *bcl-xl* promoter (226-915 nt; Gene Bank Accession No. D30746) was cloned into pGL3-Basic Luciferase Vector (Promega Corp., Madison, WI) at *Xho*I and *Hind*III cloning sites (Sevilla *et al.*, 1999). The promoter *cis*-elements were determined by TRANSFEC v4.0 Program (TESS: Transcription Element Search System, University of Pennsylvania) and two transcription initiation sites were identified.

Cloning of pBcl-xLP Deletion Constructs

PCR was used to make nine sequential deletion pBcl-xLP constructs. The full-length pBcl-xLP(−53+647) was used as template for PCR with specific primers that amplified the appropriate regions resulting in the deletion constructs. The primers used for sequential deletion constructs were pBcl-xLP(−28,+707) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-CCCAAGC TTGTCCAAAACACCTGCTCA-3'; pBcl-xLP(−28,+542) sense: 5'-CCGCTCGAGCCACC TCCGGGAGAGTACTC-3', anti-sense: 5'-CCCAAGCTTCCAGAACTGGTTTCTTTGTGG-3'; pBcl-xLP(−28,+462) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-CCCAAGCTTCCAGTGGACTCTGAATCTCCC-3'; pBcl-xLP(−28,+375) sense: 5'-CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-

CCCAAGCTTCCCCCGCCCCCACTCCCGCTC-3'; pBcl-xLP(−28,+342) sense: 5'-
CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-
CCCAAGCTTTACATTCAAATCCGCCTTAG-3'; pBcl-xLP(−28,+282) sense: 5'-
CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-
CCCAAGCTTTCACAGGTCG GGAGAGGAGG-3'; pBcl-xLP(−28,+222) sense: 5'-
CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-
CCCAAGCTTGCTGGCAAAAAAACCAGCTC-3'; pBcl-xLP(−28,+132) sense: 5'-
CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-
CCAAGCTTAACCAGCCCCCTCG TTGCT-3'; pBcl-xLP(−28,+42): sense: 5'-
CCGCTCGAGCCACCTCCGGGAGAGTACTC-3', anti-sense: 5'-
CCCAAGCTTCCCCTCTCTTGCACGCCC-3'. The PCR cycles were: 1 cycle of 94°C for
3 min; 32 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min; 1 cycle of 72°C for 10
min. The PCR products were gel extracted with the QIAXEXII Gel Extraction Kit (Qiagen,
Inc., Valencia, CA) and sub-cloned into the pGL3-Basic Luciferase Vector (Promega Corp.,
Madison, WI) at *Xho*I and *Hind*III cloning sites.

Promoter Activity Assays

Wild-type, mutant, or deletion constructs of pBcl-xLP were co-transfected with pCMV-β-
galactosidase plasmid into MCF10A cells with FuGENE-6.0 Transfection Reagent (Roche
Diagnostics, Indianapolis, IN). To determine the effect of CSC, cells were treated with
different concentrations of CSC for indicated periods of time as shown in the respective
figure legends. Cells were harvested, lysates prepared, and processed for promoter activity
by measuring luciferase activity. The transfection efficiency of the promoter constructs were
normalized with β-galactosidase activity.

Site-directed Mutagenesis

The QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to
mutate two C/EBP sites on the pBcl-xLP construct. Primers specific for the *cis*-element
mutations were used to perform PCR. C/EBP site-I sequence,
AAAAA<u>CAAAAACC</u>AACTAAA, was mutated to AAAAA<u>GGGCCCAA</u>AATAAA, using
the following primers: sense, 5'-
TGGTGCTTAAATAGAAAAAAGGGCCCAAAACTAAATCCATACCAGCCACCT-3';
anti-sense, 5'-
GGTGGCTGGTATGGATTTAGTTTTGGGCCCTTTTTTCTTTTTTCTATCTATTTAAGC
ACCA-3'. The C/EBP site-II sequence, CCTGAGC<u>TTCGCA</u>ATTCCTG, was mutated to
CCTGAGC<u>CACAGC</u>ATTCCTG with the following primers: sense, 5'-
AGCAAGCGAGGGGGCTGGTTCCTGAGCCACAGCATTCCTGTGTCGCCTTCT-3';
anti-sense, 5'-
AGAAGGCGACACAGGAATGCTGTGGCTCAGGAACCAGCCCCCTCGCTTGCT-3'.
The PCR amplified products were digested with *Dpn*I to degrade the template DNA and
transformed into XL-1 Blue Super-competent Cells (Strategene, La Jolla, CA). Positive
clones were confirmed with sequencing and amplified for isolation of the plasmid.

Electrophoretic Mobility Shift Assay (EMSA)

Single-stranded oligonucleotides were annealed to produce double-stranded oligonucleotides for EMSA analysis. The oligonucleotides used were C/EBP-binding site-I wild-type sense: 5'-AAAAA<u>CAAAAACC</u>AACTAAA-3', anti-sense: 5'-TTTAGTTGGTTTTTGTTTTT-3'; C/EBP-binding site-I mutant sense: 5'-AAAA**GGGCCCAA**AAACTAAA-3', anti-sense: 5'-TTTTAGTTTGGGCCCTTTTT-3'; C/EBP-binding site-II wild-type sense: 5'-CCTGAGC<u>TTCGCA</u>ATTCCTG-3', anti-sense: 5'-CAGGAATTGCGAAGCTCAGG-3'; C/EBP-binding site-II mutant sense: 5'-CCTAGC**CACAGC**ATTCCTG-3', anti-sense: 5'-CAGGAATGCTGAGGCTCAGG-3'. The underlined nucleotides are the core of the C/EBP consensus sequence and the bold nucleotides were mutated. Double-stranded oligonucleotides were end-labeled with ^{32}P-γ [ATP] by T4 polynucleotide Kinase (New England BioLabs, Inc., Ipswich, MA). EMSA and super-shift analysis was performed with nuclear extracts (Shapiro *et al.*, 1988) as described in earlier studies (Jaiswal *et al.*, 2006). The antibodies specific to C/EBPα, C/EBPβ or C/EBPδ proteins from Santa Cruz Biotechnology, Santa Cruz, CA, were used for super-shift analysis.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP analysis was carried out using a kit from Upstate Biotechnology (Lake Placid, NY) as described in our earlier studies (Jaiswal *et al.*, 2006). The lysate was sonicated on ice for 4 cycles of 30 sec with 20 sec intervals using a Branson Sonicator 450 (Branson Power Company, Danbury, CT) at a 5% duty cycle, 20% constant maximal power, and with a control output of five. Anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used to immunoprecipitate the DNA-protein complexes which were then eluted and heated to reverse the cross-linkages. DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The isolated DNA was used in PCR reactions with primers specific to C/EBP site-II on the pBcl-xLP. The primers were C/EBP site-II sense: 5'-CGGGTGGCAGGAGGCCGCGGC-3' and C/EBP site-II anti-sense: 5'-AACTCAGCCGGCCTCGCGGTG-3', resulting in a 190 bp product.

Overexpression of C/EBP

MCF10A cells were plated at the density of 0.6×10^6 cells per 60 mm tissue culture plate and allowed to attach overnight. The next day, 2 μg of either C/EBPβ overexpression construct or empty pCDNA3.1 vector was transfected into the cells with 9 μl of FuGENE 6.0 Transfection Reagent (Roche Applied Bioscience, Indianapolis, IN) as previously described. Cells were treated with CSC 24 h post transfection for an additional 24 h and cell lysate was prepared for luciferase analysis or Western analysis.

Acknowledgements

Abbreviations

C/EBP	CCAAT/enhancer binding protein
CSC	cigarette smoke condensate
ChIP	chromatin immunoprecipitation
ER-	estrogen receptor negative
EMT	epithelial mesenchymal transition

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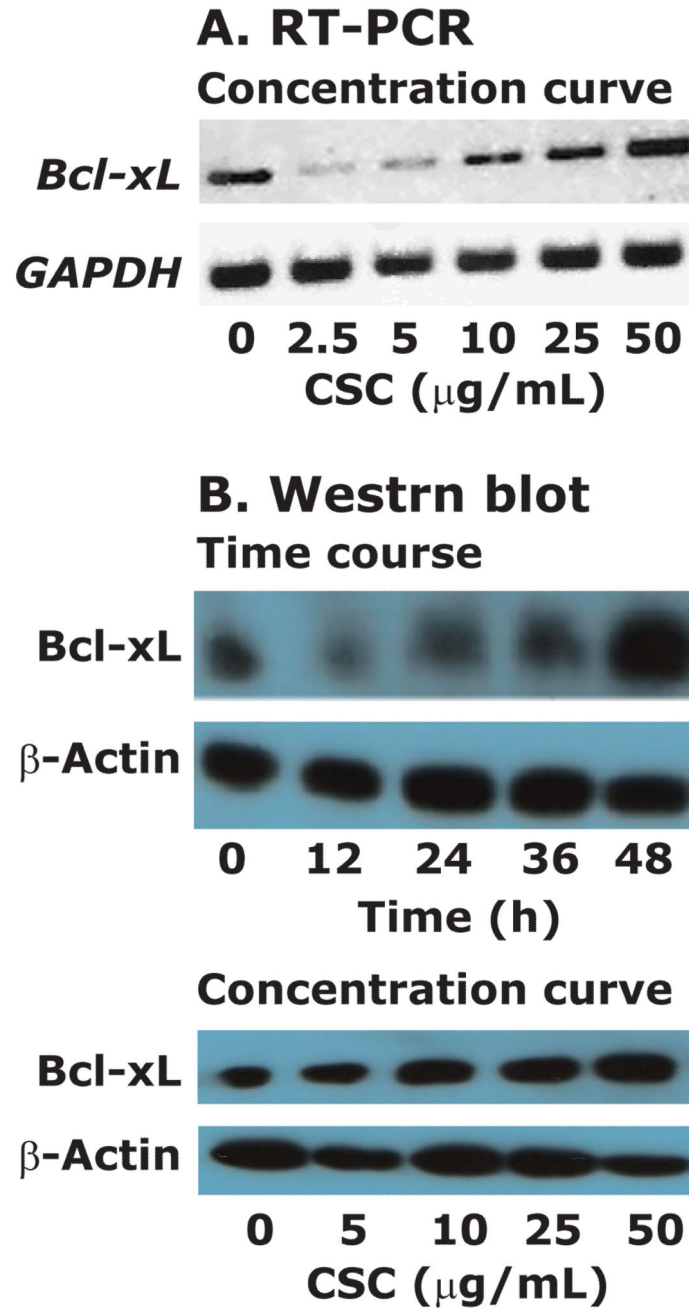
A. RT-PCR
Concentration curve

Bcl-xL

GAPDH

0   2.5   5   10   25   50
CSC (μg/mL)

B. Westrn blot
Time course

Bcl-xL

β-Actin

0   12   24   36   48
Time (h)

Concentration curve

Bcl-xL

β-Actin

0   5   10   25   50
CSC (μg/mL)

Fig. 1. *Bcl-xl* **mRNA and protein levels are induced in MCF10A cells treated with CSC**
***Paenl A*, mRNA levels.** Total RNA was isolated from cells treated with increasing
concentrations of CSC for 24 h. RT-PCR was performed with primers specific to the *bcl-xl*
cDNA sequence. GAPDH primers were used on the same samples as a loading control.
Panel B, protein levels. For the time course studies, cells were treated with 25 μg/mL of
CSC for various time points (upper panel). For the concentration curve, cells were treated
with increasing amounts of CSC for 24 h (lower panel). β-Actin levels were used as a
loading control.

```
                 5`-ct ccctgcgtcc ctcactgaaa ccttgaaccc cattgagaag -119
                                                             NF-κB

      tccctttagg gtttcggacg cctccacctc accctgggct ggtgcttaaa -69
                                         AP2

      tagaaaaaag aaaaacaaaa accaactaaa tccataccag ccacctccgg -19
                          HNF     C/EBP

      gagagtactc ctggctccca gtaggaggcg gagagccaag gggcgtgcaa +32
                          →+1                          Oct1

      gagagagggg gctgggctcc cgggtggcag gaggccgcgg ctgcggagcg +82
                      AP-2                       AP-2   →+78

      gccgccctcg atccgggcga tggaggagga agcaagcgag gggctggtt +132
        Sp1                  GATA                AP-2

      cctgagcttc gcaattcctg tgtcgccttc tgggctccca gcctgccggg +182
               C/EBP

      tcgcatgatc cctccggccg gagctggttt ttttgccagc caccgcgagg +232


      ccggctgagt taccggcatc cccgcagcca cctcctctcc cgacctgtga +282
              AP-1         GATA      AP-2            AP-2

      tacaaaagat cttccggggg ctgcacctgc ctgcctttgc ctaaggcgga +332
              GATA                USF               C/EBP    Sp1

      tttgaatgta ggtggtgcgg gggagcggga gtgggggcgg ggggactgc +382
                                           Sp1        NF-κB

      ccagggagtg actttccgag gaaggcattt cggagaagac gggggtagaa +432
                  NF-κB                  STAT

      aaggctggtg ggagattcag agtccactgg tgctttcgat ttgacttaag +482
                  GATA                             C/EBP

      tgaagtatct tggaacctag acccagacct tcgtaagacc cacaaagaaa +532
            GATA                      C/EBP

      ccagttctgg tacctggagg gggaatggaa tttttagggt aaatggcatg +582
                              NF-κB

      catattaatt attttttttt tcctgaatct ctttctctcc cttcagaatc +632
                                      GATA              GATA

      ttatcttggc tttggatctt agaagagaat cactaaccag agacgagact +682


      cagtgagtga gcaggtgttt tggac-3`                        +707
```

Fig. 2. Sequence of the cloned human *bcl-xl* promoter, pBcl-xLP

Nucleotides 226 to 915 of the human *bcl-xl* promoter were cloned into a pGL3-Basic Luciferase Vector and was named pBcl-xLP. The pBcl-xLP contains putative binding sites for several transcription factors and the transcriptional initiation sites are located at +1 and +78.
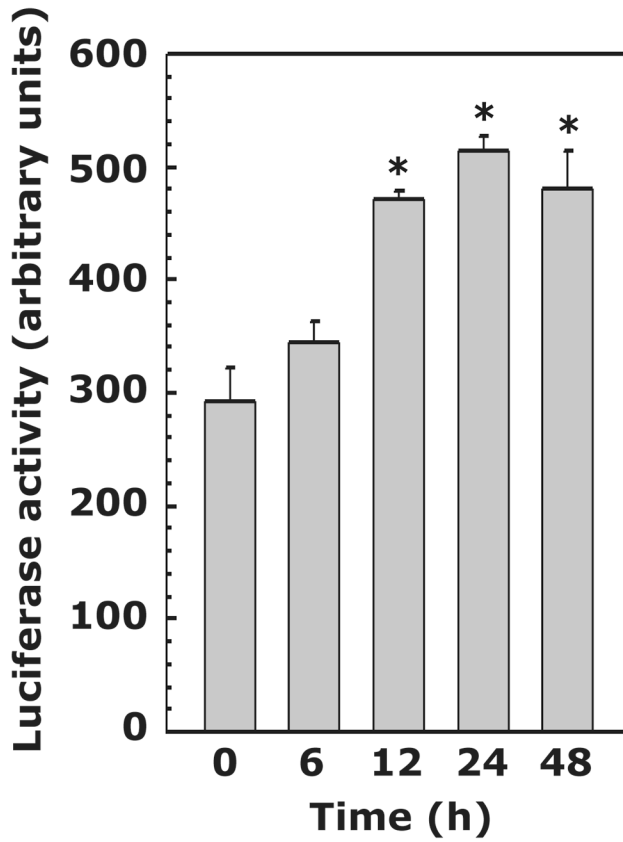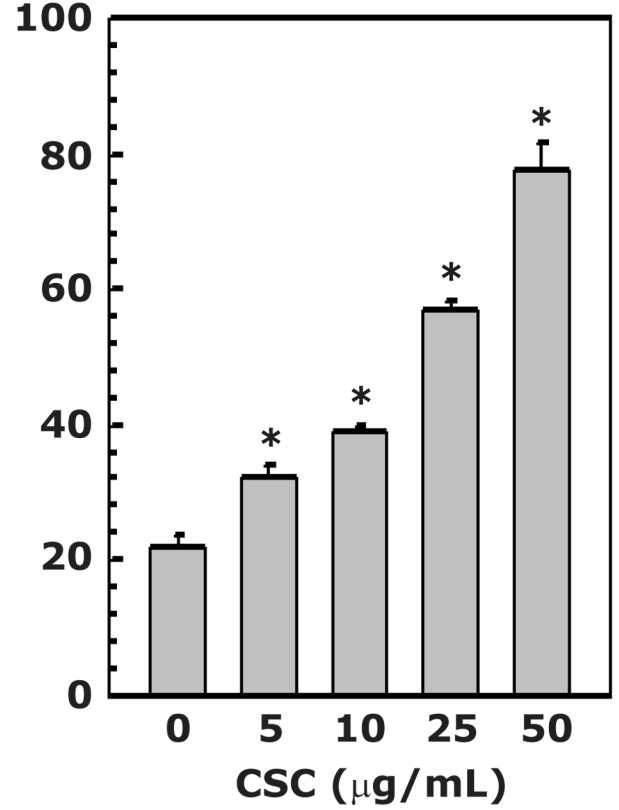
A. Time course

B. Concentration curve

Fig. 3. CSC treatment induces pBcl-xLP promoter activity *in vitro*
The pBcl-xLP construct was transfected into MCF10A cells and subsequently treated with
CSC. Cells were harvested for a time course (***Panel A***) and a concentration curve (***Panel B***)
promoter activity was measured with luciferase assays and results were expressed as mean ±
SE of triplicate experiments which are representative of three different experiments. *,
Significantly different as compared to control.

A. Structure of pBcl-xLP
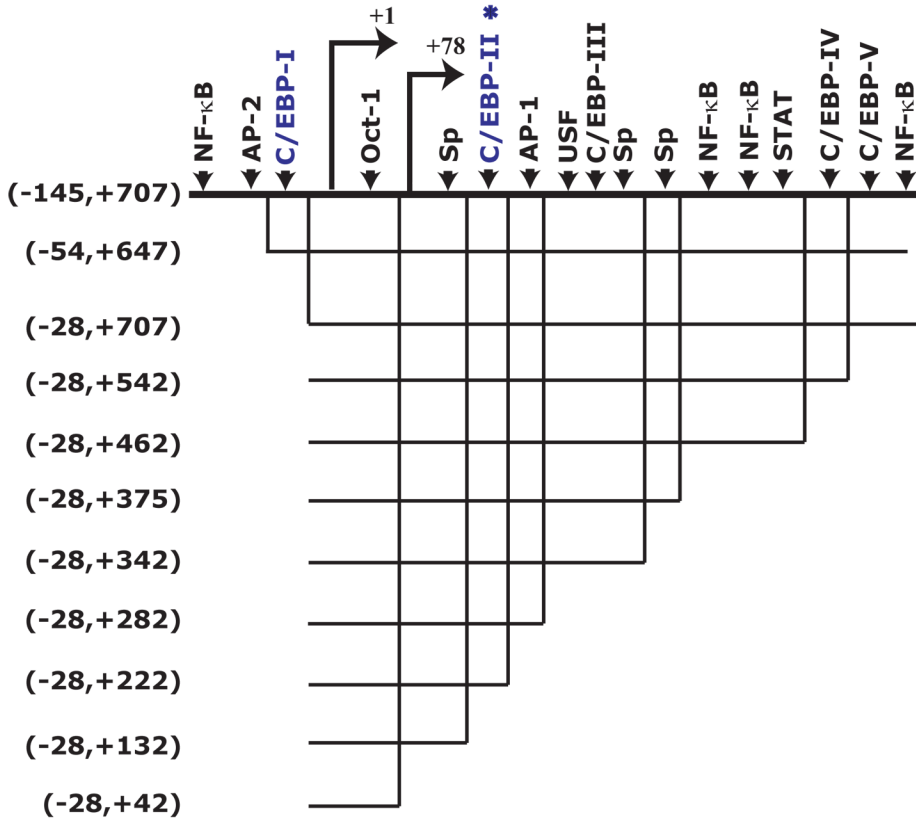
B. CSC-induced pBcl-xLP activity
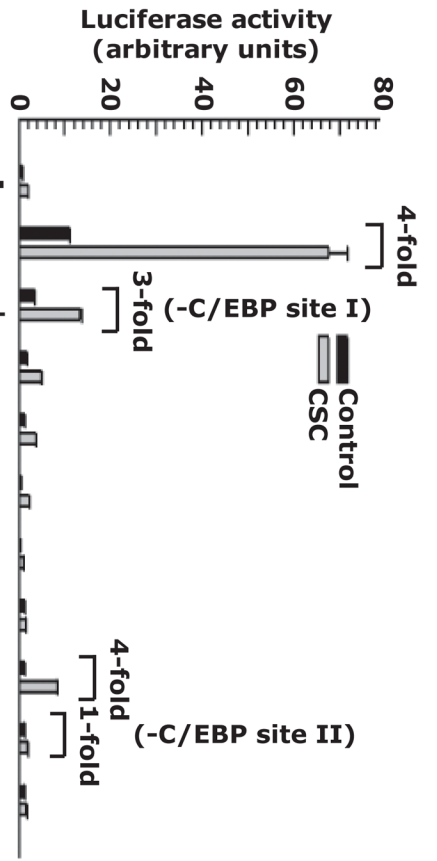
Fig. 4. The pBcl-xLP promoter contains CSC-responsive *cis*-elements
Panel A, The basal promoter construct was identified as pBcl-xLP(−54+647). Nine pBcl-xLP deletion constructs (labeled according to their lengths) were designed to sequentially delete putative *cis*-elements. Arrows indicate the transcription initiation sites. ***Panel B,*** for the determination of the CSC-responsive *cis*-elements on the pBcl-xLP, luciferase assays were used to measure pBcl-xLP promoter activity in MCF10A cells separately transfected with each construct and then treated with CSC. Results were expressed as mean ± SE of triplicate experiments which are representative of three different experiments. *, C/EBP consensus binding site.

A. Promoter activity
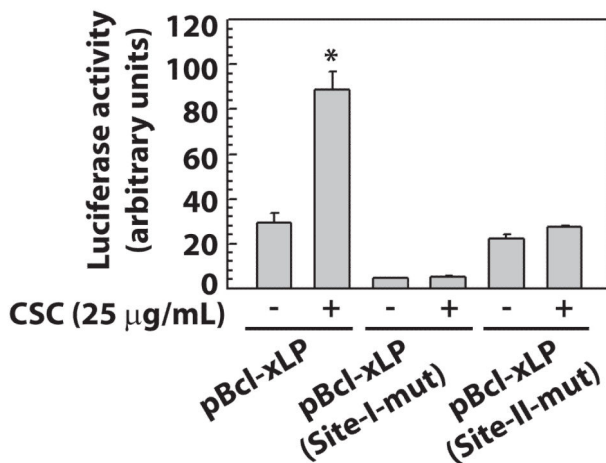
B. Western blot (time course)
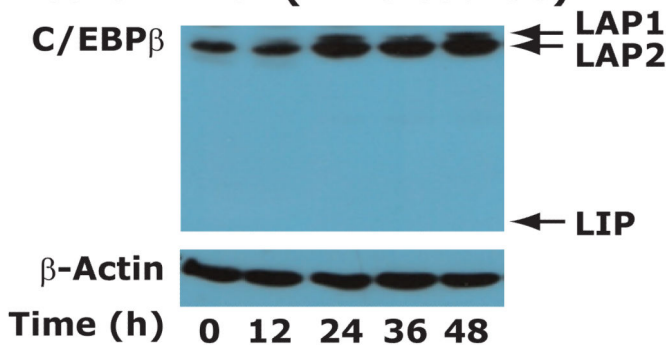
C. Western blot (concentration curve)
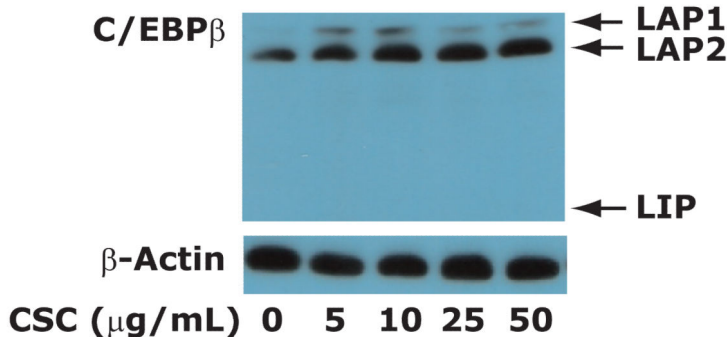
Fig. 5. *Panel A,* **shows the effect of C/EBP-binding sites of pBcl-xLP on CSC-induced promoter activity**

Wild-type pBcl-xLP and C/EBP-binding site-I and II mutant pBcl-xLP plasmids were separately transfected into MCF10A cells. The transfected cells were then treated with CSC and the promoter activity was analyzed with luciferase assays. Results were expressed as mean ± SE of triplicate experiments which are representative of three different experiments. *Panel B and C***, indicate that C/EBPβ protein levels are induced in MCF10A cells treated with CSC.** MCF10A cells were treated with 25 μg/mL of CSC for various time points. For the concentration curve, cells were treated with increasing amounts of CSC for

24 h. The protein levels of hLAP1, hLAP2 and hLIP were determined by Western blot analysis. β-Actin levels were used as a loading control. *, Significantly different as compared to control.
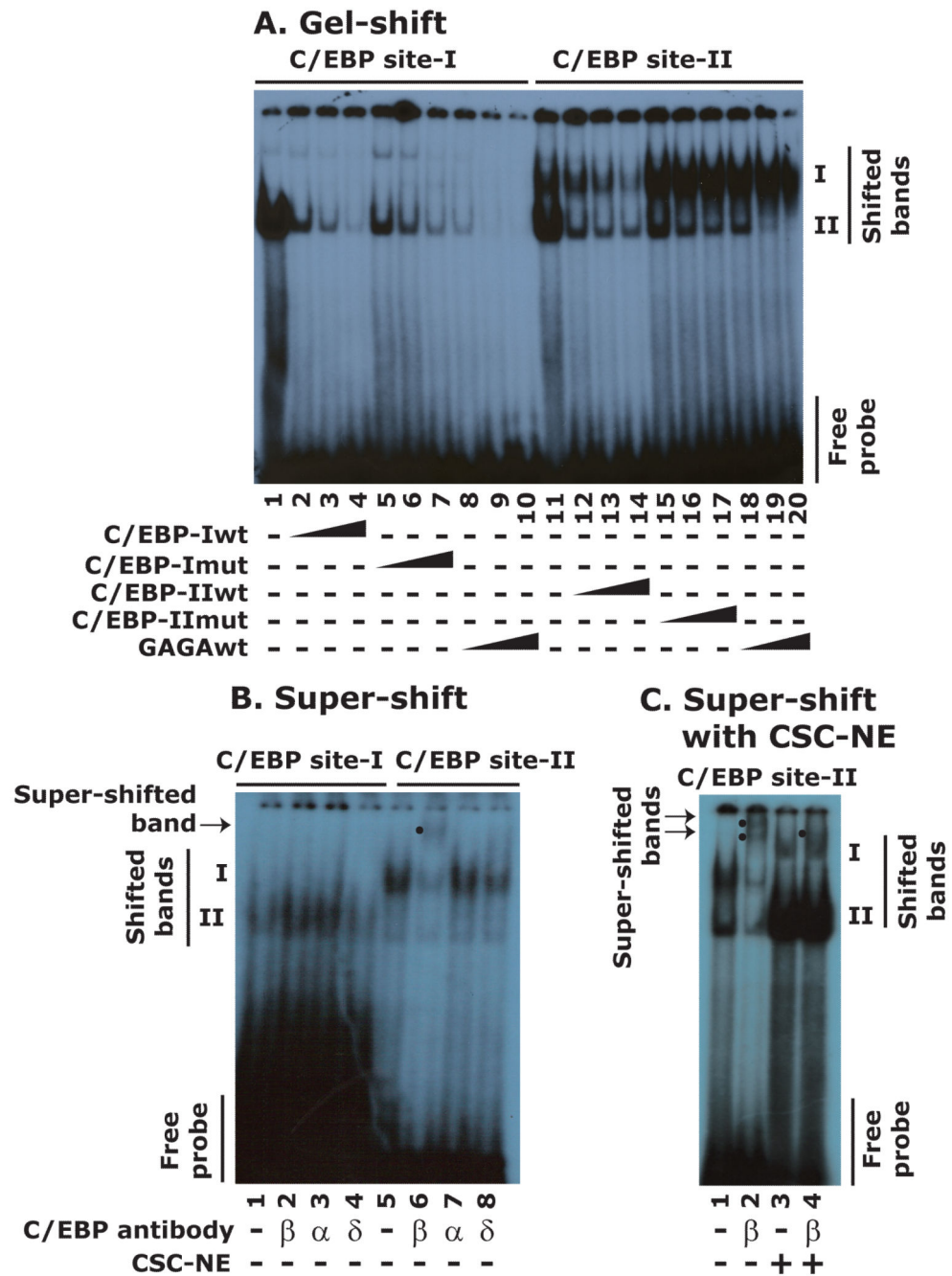
Fig. 6. C/EBPβ binds the *bcl-xl* promoter *in vitro*

[32]P-labelled C/EBP-binding site-I or ^{32}P-labelled C/EBP-binding site-II oligonucleotides were incubated with MCF10A nuclear extract. ***Panel A,*** for competition experiments, 2.5, 5, and 10-fold excess of the appropriate unlabelled wild-type or mutant C/EBP-binding site oligonucleotides were added to the indicated lanes. Unlabelled GAGA oligonucleotides were added to the lanes indicated to determine binding specificity. ***Panel B,*** super-shift analysis of C/EBP-binding site-II was carried out by adding antibodies specific to C/EBPβ, α, or δ to the reaction mixtures as the lanes indicate. ***Panel C,*** the effects of CSC treatment

on super-shift analysis were analyzed by adding anti-C/EBPβ antibody to reaction mixtures using MCF10A nuclear extract from control or CSC treated cells. The super-shifted bands are shown with arrows and dots.
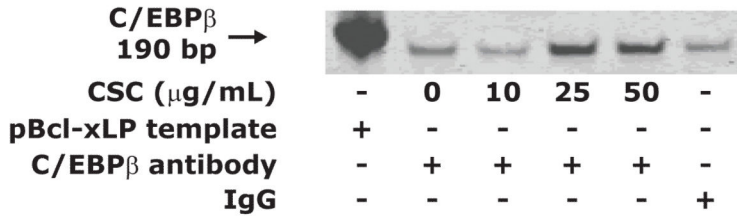
A. ChIP analysis

C/EBPβ 190 bp →
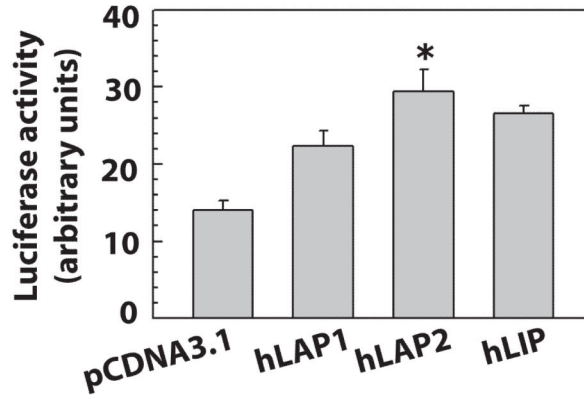
| CSC (μg/mL) | - | 0 | 10 | 25 | 50 | - |
pBcl-xLP template	+	-	-	-	-	-
C/EBPβ antibody	-	+	+	+	+	-
IgG	-	-	-	-	-	+

B. Promoter activity

C. Western blot
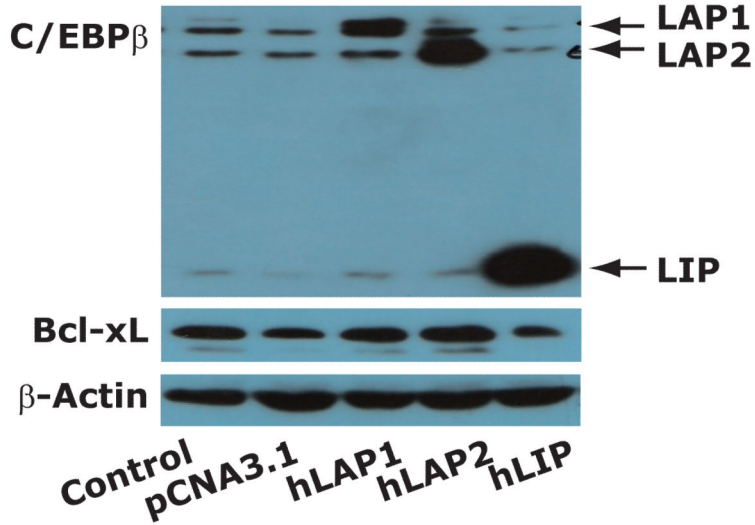
Fig. 7. *Panel A*, shows a ChIP analysis indicating that C/EBPβ is present on the *bcl-xl* promoter of MCF10A cells *in vivo*

ChIP was performed on MCF10A cells treated with increasing concentrations of CSC for 24 h. An anti-C/EBPβ antibody was used to immunoprecipitate the DNA-protein complexes. PCR was performed on the isolated DNA with primers specific for C/EBP site-II on the pBcl-xLP promoter. *Panel B and C,* **show that effect of overexpression of C/EBPβ on the pBcl-xLP promoter activity and Bcl-xL protein levels in MCF10A cells.** Human C/EBPβ overexpression constructs, LAP1, LAP2, and LIP were co-transfected with the pBcl-xLP

promoter into MCF10A cells. The promoter activity was determined with luciferase activity and results were expressed as mean ± SE of triplicate experiments which are representative of three different experiments (***Panel B***). Western analysis of C/EBPβ was used to confirm the overexpression of the appropriate construct and changes in Bcl-xL expression were assessed (***Panel C***). β-Actin levels were used as a loading control. *, Significantly different as compared to pCDNA3.1 vector control.

A. C/EBP site-I-mut activity
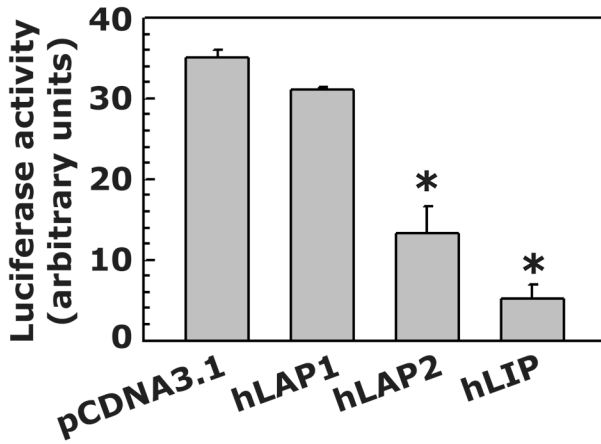
B. C/EBP site-II-mut activity
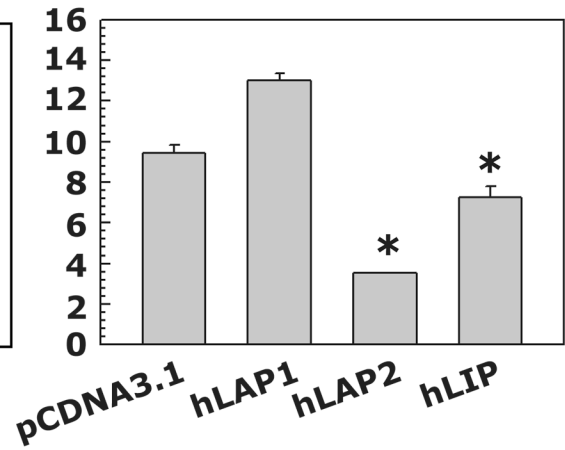
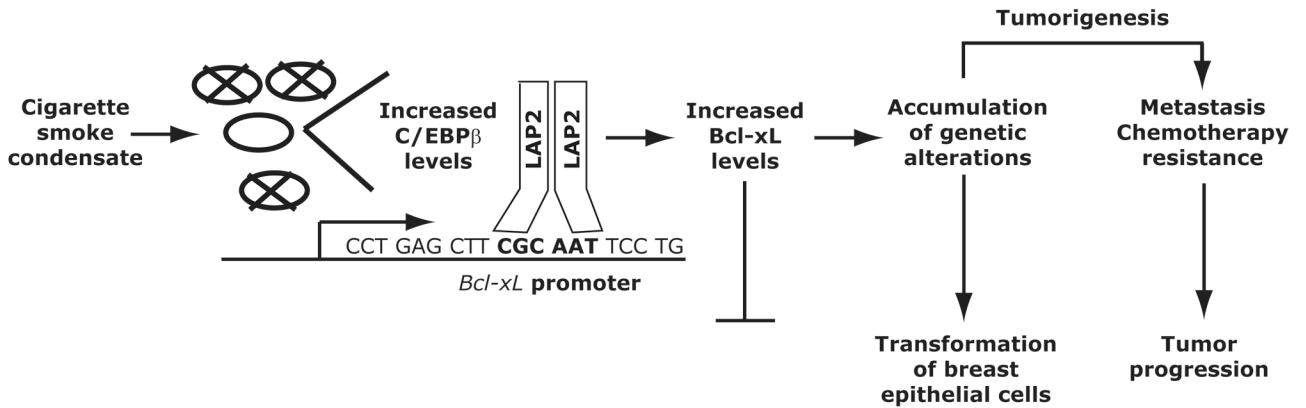## C. Model for CSC-induced *Bcl-xL* gene expression

Fig. 8. *Panel A and B,* **show that site-directed mutagenesis of C/EBP-binding sites on the pBcl-xLP attenuate the C/EBPβ-induced activation of the *bcl-xl* promoter**
C/EBPβ overexpression constructs were co-transfected with each C/EBP mutant construct. Promoter activity was analyzed with luciferase activity and results were expressed as mean ± SE of triplicate experiments which are representative of three different experiments. *, significantly different as compared to pCDNA3.1 vector control. ***Panel C,* a model of CSC-induced C/EBPβ upregulation of Bcl-xL in MCF10A cells.** Exposure of CSC to MCF10A cells causes DNA damage and most cells die. The surviving cells display increased levels of C/EBPβ by an unknown mechanism. C/EBPβ-LAP2 homodimers form and bind to C/EBP-binding site-II on the *bcl-xl* promoter, positively activating its transcription. Increased levels of Bcl-xL protein prevents damaged cells from being removed by apoptosis. Persistent DNA damage in these cells leads genetic alterations, transformation of normal epithelial cells, and eventually breast carcinogenesis. During carcinogenesis, Bcl-xL expression is linked to metastasis and resistance to chemotherapy which affect tumor progression.