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Role for Bcl-x_L in the Regulation of Apoptosis by EGF and TGFβ1 in c-myc Overexpressing Mammary Epithelial Cells

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We previously showed that TGFα synergizes with c-myc in mammary tumorigenesis through inhibition of Myc-induced apoptosis. We therefore examined the effects of growth factors on apoptosis induction in several cell lines from MMTV-*myc* mammary tumors. When EGF was withdrawn or TGFβ1 was added, cells became apoptotic after 15 h (by ELISA and morphology). Northern and Western analysis revealed high levels of Bax and p53, and low or undetectable levels of Bcl-2 and Bcl-x_S under all treatment conditions. In contrast, Bcl-x_L expression was highest in the presence of EGF or TGFα, with a significant reduction upon removal of EGF or exposure to TGFβ. In mouse mammary tumors, the relative Bcl-x_L/Bax ratio was higher in TGFα/Myc double transgenics than in Myc single transgenics, in agreement with the *in vitro* data. Our results suggest a role for Bcl-x_L in the regulation of apoptosis by EGF and TGFβ in mammary epithelial cells.

The proto-oncogene c-myc encodes a transcription factor which forms a heterodimer with Max (1). Although the Myc/Max targets are not well defined, Myc is believed to have an important regulatory function in cell proliferation. Myc expression is tightly regulated and correlated with the proliferative state of the cell. Reduced c-myc levels due to disruption of one allele results in a lengthened G1 phase (2), while inhibition of c-myc expression blocks cell cycle progression and leads to G1 arrest (3). Conversely, cells which constitutively express c-*myc* cannot arrest in G1 and thus continue to proliferate or undergo apoptosis (4).

Activation of c-myc is thought to play a role in the development of breast cancer since it is commonly amplified and/or overexpressed in human breast tumors (5, 6). c-myc amplification is associated with a high proliferation index in mammary tumors and is correlated with poor prognosis. In addition, Myc confers tumorigenicity when overexpressed in the mammary gland of transgenic mice (7). Recent results indicate that overexpression of both c-*myc* and TGFα cooperate strongly in mammary tumorigenesis (8, 9). The contribution of TGFα may be due, at least in part, to the suppression of myc-induced apoptosis (10).

Apoptosis is regulated by the bcl family of proteins which either promote or inhibit cell death (11). The death suppressor Bcl-2, initially recognized for its role in lymphoma, has since been found to be highly expressed in a variety of tumors (12). When overexpressed, Bcl-2 can protect cells from many apoptotic signals, including unregulated myc expression (4, 11, 12). Bcl-x is a unique family member in that the mRNA can be alternately spliced to produce 2 different proteins: a death suppressor (Bcl-x_L) and a death inducer (Bcl-x_S). The suppressive activity of Bcl-2 and Bcl-x_L can be modulated by death inducers such as Bax, a family member which forms heterodimers with the two former proteins. The ratio of Bcl inducers to suppressors

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determines the fate of the cell (11). Bcl-x and Bax are both expressed in breast tissue and play an important role in the normal apoptotic process of mammary gland involution (13, 14).

The tumor suppressor p53 has also been implicated in the regulation of apoptosis and, like Myc, can regulate growth as well as death. Expression of p53 leads to cell cycle arrest or apoptosis, depending on the cell type and environment. In many, but not all systems, p53 activity is required for apoptosis. In the mammary gland, apoptosis can occur in the absence of p53 (14, 15), but it is not known whether myc-induced apoptosis requires wild type p53 in that cell type.

For this study, we wished to investigate the regulation of apoptosis in mammary epithelial cells which overexpress c-myc. Mammary tumors which arise in MMTV-myc transgenic mice, as well as epithelial cell lines derived from such tumors, both show a high propensity to undergo apoptosis (10). However, mammary tumors and cell lines which express both Myc and TGFα exhibit very low levels of apoptosis and therefore grow much faster *in vitro* and *in vivo*. Furthermore, apoptosis in the Myc single transgenic cell lines can be inhibited by exogenous TGFα or EGF and accelerated by the growth inhibitor TGFβ. We therefore examined expression of apoptotic pathway genes in the presence or absence of those growth factors.

MATERIALS AND METHODS

Transgenic mice. The TGFα transgenic mice (MT100) were provided by Dr. Glenn Merlino (NIH, Bethesda, MD). The c-Myc mice (MMTV-c-*myc* M) were developed by Dr. Philip Leder (Harvard Medical School, Boston, MA) and obtained from Charles River Breeding through a breeding license with DuPont. Double transgenic mice were generated as described previously by mating the TGFα strain to the c-Myc strain (8). Mammary tumors were excised and frozen at -70°C as they spontaneously arose in each strain.

Cell lines. The cell lines Myc#83, Myc#7, and Myc#9 were established from mammary tumors of single transgenic mice as previously described (10). The cells were routinely grown in IMEM (Gibco-BRL) containing 2.5% FCS, 10 ng/ml /EGF (Upstate Biotechnology Incorporated) and 5 μg/ml insulin (Biofluids).

Cell death ELISA. Cytoplasmic nucleosomal DNA fragments were detected using an apoptotic cell death ELISA (Boehringer Mannheim) with antibodies directed against histones and DNA, as previously described (66). Cells were plated in 12-well plates (6.7×10^4 cells/well) and were treated for 3-48 hours prior to lysis with the following growth factors: EGF (10 ng/ml), TGFβ1 (100 pM, R & D Systems), or EGF plus TGFβ.

Northern analysis. Cells were plated (5×10^5 cells/ 60 mm plate) and treated as above for 24 h. Total RNA was harvested by the guanidine thiocyanate-acid phenol method. Total RNA (15 μg) was separated on 1% agarose-formaldehyde gels and transferred to nylon membranes (Amersham). Blots were sequentially hybridized overnight with the following ^{32}P-labeled, random-primed murine probes: bcl-2 (nt 1635-1945), bcl-x_{L+S} (mRNA nt 110-394), bax (mRNA nt 138-389), and p53 (nt 97-1407).

RT-PCR. The relative amounts of bcl-x_L and bcl-x_S mRNA were measured by an RT-PCR assay. One μg RNA from each sample was reverse transcribed with random primers and the cDNA for bcl-x_{L+S} was amplified for 31 cycles using a pair of primers that amplify the nucleotide sequence containing the region differentially spliced in the bcl-x_L and bcl-x_S mRNAs. 5′ primer (mRNA nt 466-488): 5′-GCG CGG GAG GTG ATT CCC ATG GC-3′; 3′ primer (nt 891-870): 5′-CAT GCC CGT CAG GAA CCA GCG G-3′. PCR products were fractionated on a 2% agarose gel and transferred to a nylon membrane which was sequentially hybridized with a ^{32}P-labeled random-primed probe for bcl-x_L (Bcl-x mRNA nt 466-891) and an oligonucleotide specific for the splice site within the 237-bp bcl-x_S product (5′-CAG AGC TTT GAG CAG GAC ACT TTT GTG G-3′).

Western analysis. Cells were plated (1.3×10^6 cells/100 mm plate) and treated as above for 24 h before lysis in RIPA buffer (PBS with 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS). Frozen mouse mammary tumors were pulverized in liquid nitrogen and homogenized in Tris-SDS (10 mM, pH 7.4, 1%). Twenty μg of protein were separated on 14% SDS-PAGE gels (or 10% for p53) and transferred to nitrocellulose. Blots were blocked with 5% milk in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris, pH 7.4, 150 mM NaCl, 0.2% Tween-20) for 1 h and then incubated in TBST with 1% BSA and the following antibodies (diluted 1/400): Bcl-x_{L+S} (S-18), Bax (N-20), Bcl-2 (N-19) (Santa Cruz Biotechnology); or p53 (Ab-1, Oncogene Sciences). Proteins were visualized with an HRP-linked second antibody (1/500 in TBST with 1% BSA) and a chemiluminescent detection system (Pierce).

RESULTS

Three mammary epithelial cell (MEC) lines derived from tumors of MMTV-myc mice were tested for their apoptotic response to EGF and TGFβ. Southern analysis demonstrated

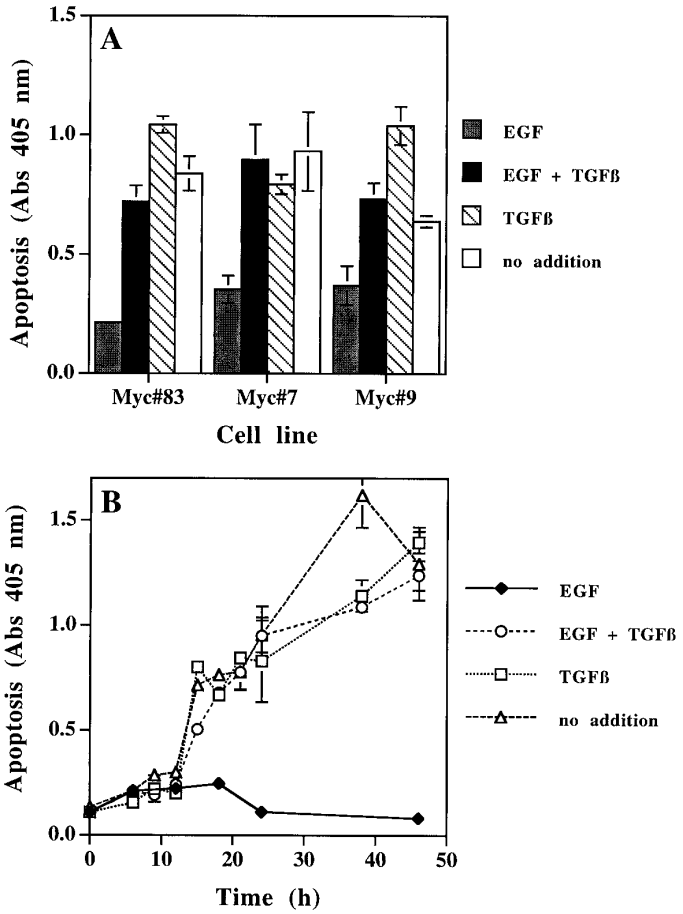

FIG. 1. Apoptosis in MECs which overexpress c-myc. (A) Three cell lines derived from mammary tumors of MMTV-myc transgenic mice were treated for 24 h with: EGF (10 ng/ml), TGFβ1 (100 pM), EGF + TGFβ, or no addition. Apoptotic DNA fragments were detected in cytoplasmic lysates via histone-DNA ELISA. n=2 (+/−SE). (B) Time course of apoptosis induction in Myc#83 cells. Cells were treated as in A and lysates were harvested at three hour intervals for ELISA. n=4 (+/−SE).

that the cell lines retained the myc transgene, and expression *in vitro* was confirmed by Northern and Western blots (not shown). Cells were treated for 24 h before preparing cytoplasmic lysates for assessing apoptosis via histone-DNA ELISA. For each cell line, the occurrence of apoptosis was lowest in the presence of EGF (Figure 1A). The level of apoptosis increased dramatically following EGF withdrawal or exposure to TGFβ. However, there was no apparent synergism for apoptosis induction between addition of TGFβ and removal of EGF.

The time course of apoptosis induction was assessed in the Myc#83 cells. At all time points, cells treated with EGF showed very low, basal levels of apoptosis (Figure 1B). In contrast, cells deprived of EGF or treated with TGFβ showed a very similar temporal pattern of cell death. They began to exhibit cytoplasmic histone-DNA complexes at about 15 hours after treatment and achieved maximal levels between 24 and 48 hours.

The ELISA results were confirmed by observing morphological changes in Myc#83 cells undergoing apoptosis (not shown). Cells grown with EGF appeared healthy and displayed

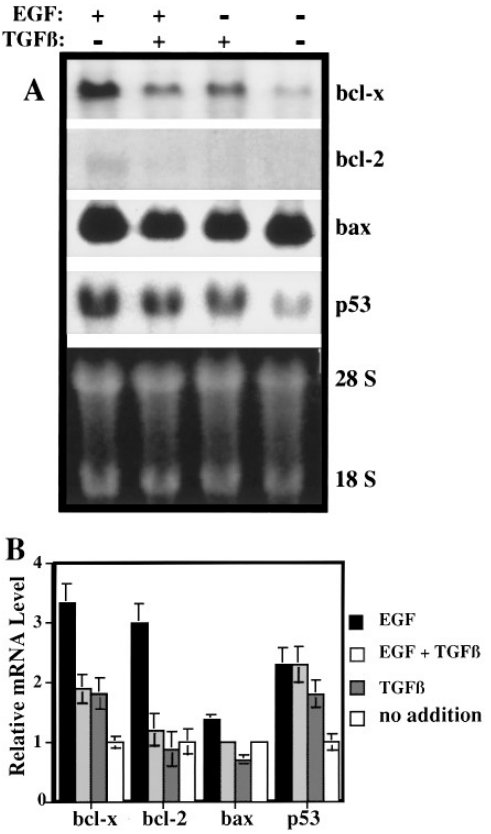

FIG. 2. Northern Analysis. Myc#83 cells were treated for 24 h as in Fig. 1A before harvesting total RNA. (A) Representative results. Northern blots were sequentially hybridized with random-primed probes for mouse bcl-2, bcl-x, bax, and p53. 28S and 18S ribosomal RNA bands are shown as a loading standard. Autoradiography exposure times were as follows: bcl-2, 7 d; bcl-x_{L+S}, 5 d; bax, 3 d; p53, 2 d. (B) Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=8 (+/−SE).

many mitotic figures. In contrast, cells exposed to TGFβ or deprived of EGF exhibited a very typical apoptotic morphology, with DNA condensation at the nuclear membrane and prominent apoptotic bodies. Furthermore, mitotic structures were rare under conditions which favored apoptosis.

Based on the results in Fig. 1B, a 24 h treatment period was chosen to assess changes in gene expression. Northern blots were sequentially hybridized with murine probes for bcl-2, bcl-x, bax, and p53 (Figure 2). Bcl-x showed the greatest variation in expression. Levels were highest in EGF treated cells and were significantly decreased following TGFβ treatment or EGF withdrawal. Expression of bcl-2 was clearly much lower than bcl-x, but the pattern of expression was similar, with the strongest signal for cells grown in the presence of EGF. In contrast, bax RNA was easily detectable and was relatively uniform across treatments. Expression of p53 RNA was also consistent across treatments, with the notable exception of EGF deprivation (50% reduction).

RT-PCR was used to distinguish the long and short forms of bcl-x RNA (Figure 3). A single band corresponding to bcl-x_L was visible on ethidium bromide stained gels of the PCR products. When the DNA was transferred to nylon and hybridized with a random-primed

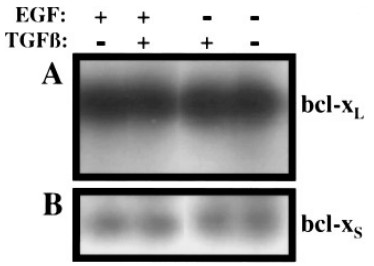

FIG. 3. Bcl-$x_{(L,S)}$ RT-PCR. RNA samples were reverse-transcribed and then amplified by PCR. The PCR products were separated on an agarose gel and transferred to nylon for Southern analysis. (A) The blot was hybridized with a random-primed probe (Bcl-x mRNA nt 466-891) (3 h exposure). (B) The blot was hybridized with an oligonucleotide probe specific for the short form of *bcl-x* (20 h exposure).

probe, a strong signal for bcl-x_L was observed. Hybridization with a bcl-x_S specific oligonucleotide revealed a much weaker band for the short form, with little fluctuation among treatments. The results suggest that the bcl-x signal observed by Northern analysis was largely due to expression of bcl-x_L rather than bcl-x_S.

Western blots were used to examine expression at the protein level (Figure 4). Bcl-x_L protein levels varied dramatically, with highest expression in EGF-treated cells. Bcl-x_L expression was similar in EGF- and TGFα-treated cells (not shown). Following TGFβ treatment or EGF withdrawal, there was an 80% reduction in Bcl-x_L protein. Analysis of lysates prepared at various time points indicated that the decrease in Bcl-x_L protein preceded the onset of DNA degradation (not shown). Neither Bcl-x_S nor Bcl-2 could be detected by

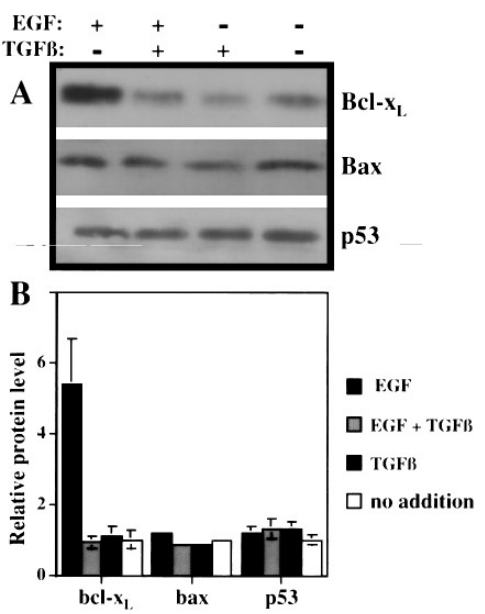

FIG. 4. Western analysis of Myc#83 cells. Cells were treated for 24 h as in Fig 1A before preparing total cell lysates. 20 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose. (A) Representative results. Individual blots were incubated with antibodies against Bcl-x, Bax, and p53. (B) Cumulative data. The mean band intensity for untreated cells was assigned a value of 1 and all other values were calculated as a relative increase or decrease. n=6 (+/−SE).

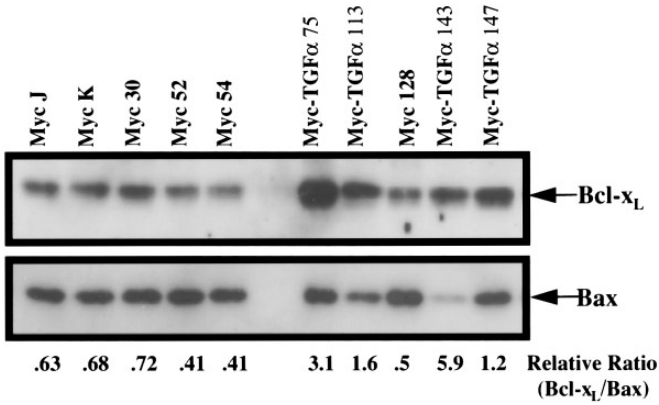

FIG. 5. Western analysis of mammary tumors from single Myc and double Myc/TGFα transgenic mice. Lysates were prepared from frozen tumors and 20 μg of protein were separated on 14% polyacrylamide gels. The blot was probed first with an antibody against Bcl-x and then stripped and re-probed with Bax antibody. Relative ratios were calculated from the band intensities as measured by densitometry.

Western analysis. Bax and p53 protein levels were relatively high and showed little variation across treatments.

The relative ratios of Bcl-x_L to Bax were compared in mammary tumors from single (MMTV-myc) or double (MMTV-myc X MT-TGFα) transgenic mice. Bcl-x_L and Bax were detected by Western analysis and relative ratios of the signal intensity for the two proteins were calculated from the values obtained by densitometry (Figure 5). Lysates from Myc single transgenic tumors consistently showed a relative ratio of Bcl-x_L to Bax of approximately 0.5 (+/− 0.06). Ratios for the double transgenic tumors were more variable, but were consistently higher than the single transgenics, with a mean value of 2.9 (+/− 1.0). Bcl-2 was not detected in the tumor lysates.

DISCUSSION

Our results suggest a role for Bcl-x_L in growth factor-mediated regulation of apoptosis in MECs which overexpress c-Myc. Bcl-x_L expression showed the greatest variation in response to EGF and TGFβ. Bcl-x_L mRNA and protein levels were dramatically elevated in Myc#83 cells grown in the presence of EGF or TGFα compared to cells which had been deprived of EGF or treated with TGFβ. Consistent with the *in vitro* results, the relative ratio of Bcl-x_L to Bax was higher *in vivo* in mouse mammary tumors which expressed TGFα and Myc together compared to those which overexpressed Myc alone.

The data indicate that MECs which overexpress c-Myc are dependent on EGF receptor signaling for survival and growth. We and others have recently demonstrated that EGF and TGFα can act as survival factors for mammary epithelial cells *in vitro* (16) and *in vivo* (10, 17). However, in the aforementioned *in vitro* study (16), much harsher conditions were used to induce apoptosis in MECs than in our study (high cell density combined with serum deprivation rather than simple withdrawal of EGF from subconfluent cells in the presence of 2.5% FCS and 10 μg/ml insulin). Many MEC lines, both human and mouse, are dependent on EGF for growth, but deprivation of EGF under normal culture conditions results in reversible growth arrest rather than apoptosis (18; personal communication, Dr. Daniel Medina, Baylor College of Medicine, Houston, TX). Those results imply that Myc-overexpressing MECs are very susceptible to the induction of apoptosis and are especially dependent on EGF receptor pathway(s) for survival. Our cell lines therefore provide an excellent model for studying

growth/survival factor regulation of Myc-induced apoptosis in MECs. The results presented here indicate that increased expression of Bcl-x_L may, at least in part, explain the mechanism by which EGF and TGFα function as survival factors.

The manner in which EGF regulates apoptosis in MECs may therefore be analogous to that observed in hematopoietic cells following cytokine withdrawal. Two recent reports implicate Bcl-x_L in the control of cell survival in normal activated T cells and in myeloid leukemia cells (19, 20). In the former study, expression of Bcl-x_L in activated T cells was significantly reduced following interleukin-2 withdrawal, while bax and bcl-2 levels did not change. The latter study showed a reduction of Bcl-x_L expression in leukemia cells following survival factor withdrawal.

Some hematopoietic cancer cells also undergo apoptosis in response to TGFβ, as in our MEC system. TGFβ induced apoptosis in leukemia cells with a concomitant decrease in Bcl-2 expression, but no change in Bax expression (21), while in lymphoma B cells, apoptosis induction by TGFβ was not accompanied by changes in Bcl-2 expression (22), suggesting that regulation of cell death by TGFβ may be cell type specific. Bcl-x_L expression was not examined in those studies.

Increased TGFβ expression has been observed in MECs which have been stimulated to undergo apoptosis (23, 24). However, it was not determined whether TGFβ secretion was required for apoptosis induction. *In vitro*, TGFβ has previously been shown to inhibit growth of MECs (25, 26), but it has not been reported to induce apoptosis. In contrast, overexpression of TGFβ1 *in vivo* has been associated with increased occurrence of apoptosis in normal MECs (27). Since the levels of growth/survival factors such as EGF and estrogen are relatively high in the mammary gland during pregnancy, the effects of TGFβ are presumed to be dominant over those of the positive survival signals, analogous to our *in vitro* observations. Our results suggest that TGFβ may block the induction of Bcl-x_L expression by survival factors like EGF.

In contrast to Bcl-x_L, expression of Bax and p53 were relatively constant in the MECs across the various treatments examined. Since c-Myc can transactivate the p53 promoter (28), and the *bax* gene promoter also contains a putative c-Myc response element (29), those two genes may be continuously activated when Myc is constitutively expressed. That could explain the propensity of Myc-overexpressing cells to undergo apoptosis when challenged with a negative growth signal. High levels of Bax may determine the ''set point'' of the cells so that any decrease in the protective Bcl family members due to removal of growth/survival signals will push cells toward apoptosis. Myc may activate the p53 and Bax pathways as a safeguard to prevent the survival of cells with oncogenic activation. The mechanism by which Myc triggers cell death is not universal however, since the induction of apoptosis by myc overexpression is dependent on wild type p53 in some, but not all systems (4, 30). The role of p53 in our MEC system is currently being examined.

Bcl-2 and Bcl-x_S apparently do not play a significant role in the MEC system examined here. The RNA levels for both were quite low and the proteins were undetectable on Western blots. Bcl-x_L and Bcl-2 regulate a common pathway (31), but the tissue specific expression patterns of the two genes do not always overlap (32), perhaps allowing for cell specific responses to different stimuli. For example, in the human breast, Bcl-2 is not expressed in functionally differentiated secretory cells, although it can be detected in non-secretory epithelial cells (33). Alternatively, the low Bcl-2 levels may be secondary to the expression of p53 in Myc#83 cells. The 5′ untranslated region of the bcl-2 gene contains a p53-dependent negative response element (34) and p53 can down-regulate bcl-2 expression in human breast cancer cells (35).

In summary, we have demonstrated a potential role for the cell survival-promoting protein Bcl-x_L in MEC apoptosis driven by c-Myc overexpression. Myc activation is common in

breast cancer, but it is well documented that Myc overexpression alone is insufficient for transformation. Since Myc can induce apoptosis as well as growth, it is likely that secondary events will block cell death, thereby allowing the stimulatory effects of Myc to predominate. We suggest that changes in Bcl-x_L expression, either directly *via* genetic alteration, or indirectly *via* increased production of survival factors, may promote tumorigenesis of cells which overexpress Myc.

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