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13. ABSTRACT ***(Maximum 200 Words)***

The c-myc oncogene is thought to play an important role(s) in the onset and progression of breast cancer, where it is amplified in approximately 15% and overexpressed in over 60% of human breast cancers. Depending upon the availability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through as yet poorly defined mechanisms. Currently the molecular mechanism by which c-Myc sensitizes cells to apoptosis is not fully understood, nor are the environmental conditions in c-Myc-overexpressing mammary epithelium that promote apoptosis versus proliferation and transformation. The molecular mechanisms by which c-Myc has been shown to mediate apoptosis are the Fas/Fas ligand pathway, p53 and mitochondrial-dependent release of cytochrome C. Previously it has been demonstrated in our laboratory that a mammary carcinoma cell line (Myc83), derived from MMTV-c-myc transgenic mouse, is sensitive to EGF withdrawal and undergoes extensive apoptosis upon inhibition of EGFR signaling. We observed that apoptosis of Myc83 under these conditions was accompanied by the up-regulation of the death receptor Fas, and its ligand FasL. The present project was funded to study the mechanism of upregulation of Fas and Fas Ligand in Myc83 cells and to determine their functional role in c-Myc-mediated apoptosis. We show that growth factor withdrawal/blockade, rFasL, and other pro-apoptotic stimuli resulted in apoptosis in Myc83. However, in work conducted in year 1 of the grant, evaluation of the mechanism responsible for apoptosis in Myc-expressing cells revealed a lack of a role for the Fas/FasL system. We now shift our focus on uncovering the functional role of a novel calcium/calmodulin kinase, whose specific expression in mammary gland development, and upregulation during late pregnancy, cancer and MMTV-*c-myc* associated mouse mammary tumors demonstrates a potential unique role in the transformation process. We aim to uncover its precise role breast cancer development.

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INTRODUCTION:

Breast cancer as a disease does not initially involve all tissues of the breast, but rather begins in the epithelial cells. The progression from a normal, healthy mammary epithelial cell to one that is transformed involves a series of molecular events including gain or expression of oncogenes, loss of tumor suppressor genes, chromosomal instability, and gain or loss of genes controlling cell cycle or cell death. Inappropriate growth of a cell, the cell's inability to respond to the cues that regulate its growth, is a hallmark of cancer onset. However, cancer onset and progression may also occur because of a disruption in the apoptotic machinery that controls programmed cell death (1,2,3). Within one cell, a disruption of any of these cellular functions may lead to initial stages of transformation. We proposed to investigate two fundamental molecular events involved in breast cancer onset and progression c-Myc-mediated oncogenesis and apoptosis. The c-Myc oncogene is paradoxical, and when amplified or overexpressed, as it is in the majority of human breast cancers, it can contribute to a number of different, seemingly opposite cellular outcomes (4). c-Myc may contribute to tumor initiation and progression by inducing cell proliferation, chromosomal instability, and abrogation of cell cycle checkpoints. Inappropriate expression of c-Myc, however, may also lead to growth arrest and apoptosis or programmed cell death in many different cell types, including mammary epithelial cells (4, 5). The ability of c-Myc to regulate the cell cycle has been studied since its identification over two decades ago, however, its role in apoptosis was only recognized in the past decade, and the mechanism(s) that c-Myc employs to exert its apoptosis effect is still largely unknown (6). Also unclear is what intracellular and pericellular environmental conditions in c-Myc-overexpressing mammary epithelium promote apoptosis versus proliferation and transformation. One factor in the cellular environment that may determine whether c-Myc-expressing cells proliferate or die is the availability of survival factors (6).

In this proposal, we aimed at uncovering the mechanisms of c-Myc-mediated apoptosis. Previously in our laboratory, mouse mammary carcinoma cells (Myc83), derived from MMTV-c-Myc tumors, demonstrated a significant degree of apoptosis upon growth factor withdrawal and pharmacological inhibition of survival signaling pathways. Similar, non-c-Myc expressing mammary epithelial cells did not demonstrate the same apoptotic phenotype under these conditions. With in the c-Myc literature, a few potential mechanisms of c-Myc-initiated apoptosis had been proposed, specifically implicated were the Fas/FasL death receptor pathway, the p53 pathway and cytocrome-c released from the mitochondria (6, 7, 8).

We observed that apoptosis in Myc83 cells, initiated via inhibition of epidermal growth factor receptor (EGFR), was accompanied by an upregulation of the death receptor Fas and its ligand FasL, at the protein level. Our proposal included aims to specifically uncover the mechanism of upregulation of Fas and FasL under conditions of apoptosis in c-Myc-expressing mammary carcinoma cells, and to determine the functional role of this death receptor pathway in c-Myc-mediated apoptosis.

This proposal served several scientific and professional purposes. Scientifically, we aimed to uncover how and when mammary carcinoma cells expressing c-Myc initiate apoptosis rather than proliferation, which had the potential to impact on the understanding of mammary carcinogenisis, and possibly to provide insight into potential pathways of intervention. The value of studying and understanding c-Myc-mediated apoptosis is heightened by the fact that the phenotypic outcome of the apoptotic process, namely cell specific and regulated cell death, exactly matches the clinical aim of cancer therapy—tumor specific cell death. Equally important, the purpose of this proposal was to provide a scientific training experience under the guidance of my mentor Dr. Robert Dickson. As these aims evolve, as they are addressed, developed, revised, explored, focused or expanded, the goal is that I too evolve as a scientist; one better equipped to ask relevant questions, choose the best methods to employ in answering them, one better able to evaluate and interpret results and ask the appropriate next question.

BODY:

Summary of Research and Training Accomplishments:

This is the annual report of training and research accomplishment between the periods of June 1, 2001-May 31, 2002 for Grant #DAMD17-01-1-0246, entitled "Fas/FasL system in c-Myc-expressing mammary carcinoma cells" conducted by the principal investigator Christine M. Coticchia under the guidance of Dr. Robert B. Dickson.

Hypothesis: In breast cancer cells, overexpression of the proto-oncogene, c-Myc has been shown to sensitize cells to apoptosis. In Myc83 cells, a mouse mammary carcinoma cell line derived from c-Myc transgenic mouse tumors, regulators of apoptosis, namely Fas/FasL, have been shown to be upregulated under pro-apoptotic conditions, such as serum and growth factor withdrawal and blockade. We first hypothesize the this upregulation of the apoptotic inducers, Fas and FasL, occurs through transcriptional mechanism(s) that respond to growth factor removal and to expression of c-Myc. We further hypothesize that this upregulation of Fas and FasL contributes to c-Myc-mediated apoptosis.

We approached each specific aim simultaneously, but initially focused in year one on the most important aim, Aim 3.

Specific Aim #3: To evaluate the functional significance of Fas/FasL expression in c-Myc expressing mammary cells.

a. We proposed to evaluate the mechanistic basis for our previously observed apoptotic phenotype, comparing exogenous recombinant FasL treatment versus endogenous upregulated FasL in Myc83 cells
b. We proposed to determine if inhibition of the Fas/FasL system abolishes c-Myc-mediated apoptosis.
c. We then proposed to investigate if conditions of cell growth are responsible for the divergent effects on cell death when comparing exogenous and endogenous FasL.

Summary of Training and Research Accomplishments for Specific Aim #3:
Independently, I reconfirmed the finding that FasL and Fas were upregulated under conditions of apoptosis in c-Myc expressing mouse mammary carcinoma cells. Specifically, under conditions where EGFR, PI3K and MEK survival signals were inhibited, Fas and FasL were upregulated. Please see Figure 1a and 1b in APPENDIX A.

We had previously stated that recombinant, exogenous FasL could induce apoptosis in Myc83 cells, thus demonstrating that the Fas pathway was intact and functional in this system. To determine if endogenous Fas and FasL upregulation was the mechanism of c-Myc mediated apoptosis in Myc83 cells upon survival factor blockade, we attempted to inhibit Fas signaling under apoptotic conditions. Using a Fas decoy receptor, the Fas receptor conjugated to the Fc portion of an IgG molecule, we blocked Fas signaling by essentially swamping all endogenous FasL with the decoy thus preventing it from initiating endogenous Fas signaling. When we did this Fas-Fc decoy experiment in Myc83 cells, we were unable to provoke survival upon EGFR signaling blockade with PD153035. Myc83 cells underwent significant apoptosis even in the presence of this Fas decoy. See Figure 2a in APPENDIX A. The Fas-Fc decoy receptor was effective at inhibiting apoptosis in Myc83 upon treatment with exogenous recombinant FasL, thus demonstrating that the Fas-Fc decoy was a biologically active reagent. See Figure 2b in APPENDIX A. To further investigate the role of the Fas/FasL pathway in c-Myc-mediated apoptosis, others in our lab performed an experiment inhibiting the Fas receptor initiator caspase, caspase-8, under

the same apoptotic conditions in Myc83. The caspase-8-inhibitor z-IETD-fmk completely inhibited recombinant FasL-induced apoptosis but was unable to prevent apoptosis induced by EGFR blockade (data not shown). These findings, taken together, began to indicate that the Fas/FasL pathway, although regulated during conditions of apoptosis, may not be functionally significant in Myc83 cells undergoing apoptosis. I then decided to seriously revisit the literature, and there I came upon two important technical articles highlighting problems arising in the FasL literature, due in part to poor quality commercial reagents (9, 10). Specifically problematic were antibodies used to detect FasL that were found to be non-specific, leading to the publication of several false positives in the FasL literature (see Supplement 1 and Supplement 2 in APPENDIX A). The technical comment revealed that the clone 33 antibody that I had purchased from Transduction Labs was detecting a 36kD protein that was not FasL. Upon their recommendations, I used a clone N-20 antibody from Santa Cruz to reconfirm my findings. The western blots revealed contradictory results. The same lysates, run on the same gel, and exposed at the same time demonstrated different FasL expressions under EGFR blockade by PD153035 (see Figure 3 in APPENDIX A). Interestingly N-20 did not detect an upregulation of FasL in Myc83 cells treated with PD153035, while Clone 33, reported as detecting a nonspecific protein, did. This finding, taken with my previous functional data, leads me to conclude that the Fas/FasL system is not functionally mediating c-Myc-induced apoptosis in Myc83 cells, most probably because FasL in not being upregulated, and therefore not activating the death pathway.

This finding allowed us to conclude that pursuit of Aim 3c, which proposed to determine if cell culture conditions were causing a variation in apoptosis caused by endogenous vs. exogenous FasL, was unnecessary.

Specific Aim #1: To determine the mechanism of Fas ligand (FasL) induction in c-Myc-expressing mammary carcinoma cells under pro-apoptotic conditions.

a. We proposed to analyze whether FasL induction was mediated at the transcriptional level, specifically we proposed to determine whether FasL mRNA levels were increased in response to growth factor withdrawal in mammary carcinoma cells that express c-Myc.
b. We proposed to determine if removal of survival factors such as serum and growth factors, removes downstream repression of FasL transcription thus inducing FasL mRNA transcription. We intended to identify which signaling and transcriptional regulatory components within the survival pathway were mediating this repression/activation.
c. We were also planning on determining if cell-cell adhesion molecules, such as cadherins, were important in mediating FasL induction. We thought to evaluate if E- cadherin, N-cadherin or cadherin-11 in c-Myc expressing cells correlated with FasL expression.

Summary of Training and Research Accomplishments for Specific Aim #1:
Before the specificity of the Clone 33 antibody was doubted, we pursued the mechanism of upregulation of FasL by focusing on Specific Aim #1 b, where we hypothesized that a family of transcription factors, the Forkhead family of transcription factors, were involved in FasL transcription. Specifically, we hypothesized that Forkhead activity was repressed in the presence of the known survival factor, Akt (11). While still using clone 33 antibody, we observed that removal of EGF or blockade of EGFR increased FasL expression. Our lab also demonstrated that Myc83 cells were shown to activate Akt upon stimulation with EGF (see APPENDIX B, paper 1). Stimulated Akt phosphorylates Forkhead transcription factors, which subsequently excludes them from the nucleus (11). The FasL promoter has three-conserved Forkhead recognition sites so it seemed reasonable at the time to begin investigating the potential role of the Akt-Forkhead pathway in FasL regulation (12).

Previously in our lab, a Myc83 cell line expressing a constitutive Akt (Myr-Akt Myc83) was created. The constitutive Akt signal provided a survival advantage and protected Myc83 cells from Myc-mediated apoptosis. I contributed to the analysis of the survival role of Akt in Myc83 cells, which lead to the submission of a paper where I am a co-author (Paper 1 in APPENDIX B). In addition to providing the figures that demonstrated the degree of apoptosis in Myc83 cells subjected to EGF withdrawal and pharmacological inhibition of survival pathways (see Paper 1, APPENDIX B figure 6a and 6b within), I also performed the Hoechst assay that revealed that Myr-Akt protected Myc83 cells from apoptosis under EGFR blockade (APPENDIX B, Paper 1and figure 7 there within). While working on these experiments I compared FasL levels in Myr-Akt Myc83 cell versus the parental Myc83 cell line. Since the presence of Akt provided such potent survival advantage to MryAkt-Myc83 cells under conditions which induced apoptosis and induced an upregulation of FasL in Myc83, I was not surprised when MyrAkt-Myc83 cell line displayed a decrease in the level of FasL compared to Myc83 (see Figure 4 in APPENDIX A).

Encouraged by these findings, I investigated whether Myc83 cells expressed any of the Forkhead family of transcription factors. While pursuing this aim, I became aware to the Clone 33 controversy, and discovered, by using Clone N-20, that FasL was not upregulated in these cells, thus negating the need to uncover the mechanism of upregulation. At that time, it became obvious that pursuing Aim 1 was unnecessary. No results gained from the Clone 33 antibody were ever published or presented in any abstract or poster.

Specific Aim #2: To evaluate the mechanism of Fas induction in c-Myc-expressing mammary carcinoma cells under pro-apoptotic conditions.

a. We proposed to analyze whether Fas induction was mediated at the transcriptional level. Specifically we wanted to determine whether Fas mRNA levels were increased in mammary carcinoma cells that express c-Myc.
b. If Fas mRNA was increased we proposed to investigate the role of p53 in mediating this transcriptional upregulation of p53.
c. If Fas mRNA was not increased as proposed in Aim 2a, we planned to evaluate the possibility that post-transcriptional modifications of Fas were responsible for its induction under pro-apoptotic conditions.

Summary of Training and Research Accomplishments for Specific Aim #2:
The investigation of the mechanism of Fas upregulation was in the initial stages when the above discoveries were made. The observation that Fas is upregulated under conditions of apoptosis in Myc83 mammary carcinoma cells is credible, to date. We believe that the Fas receptor is certainly upregulated under growth factor deprivation and Blockade (see Fig 1b, APPENDIX A). This death receptor system is function and capable of responding to exogenous, recombinant FasL. Fas and FasL, however are not the mechanism of Myc-mediated apopotosis in mouse mammary carcinoma cells and therefore the we determined that uncovering the mechanisms of Fas upregulation was not a priority since this pathway is not of functional significance in Myc-mediated apoptosis.

CONCLUSION:

The Fas/FasL system in c-Myc-expressing mouse mammary carcinoma cells are present and functional. The Fas receptor is upregulated under conditions which induce apoptosis in Myc83 cells, namely growth factor deprivation and EGFR blockade. Its ligand, FasL, however is not upregulated under conditions of apoptosis as previously thought. The Fas/FasL system in not functionally significant in c-Myc mediated

apoptosis, as inhibition of the pathway with a decoy Fas receptor did not protect Myc83 cells from apoptosis.

Revised research aims are reported in the requested Revised Statement of Work document included at the end of this report.

REQUESTED REVISED STATEMENT OF WORK:

Hypothesis: We propose that PNCK, the pregnancy upregulated nonubiquitous, CaM kinase has an anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: We aim to clone the human PNCK gene and functionally characterize its role in mammary carcinoma cells.

Aim 1a: Cloning of the human PNCK gene.

Aim 1b: Transiently and stabally express PNCK, and PNCK mutants in human mammary carcinoma cells compared to empty vector control.

Aim 1c: Functionally determine the effect of PNCK expression and PNCK mutants on cell proliferation and apoptosis *in vitro.*

Aim 2: We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Introduction: In the pursuit of uncovering molecular pathways responsible for mediating c-Myc's apoptotic effect, as alternatives to the Fas/FasL system, our lab uncovered several pathways which were responsible for inhibiting c-Myc- mediated apoptosis. One of the pathways which provided a strong survival signal in our model (Myc83, mouse mammary carcinoma cells from MMTV-*c-myc* tumor) was the PI3K/Akt pathway. Not only did EGF stimulation of Myc83 cells result in the immediate activation of Akt, but this activation prevented apoptosis of these cells. When a constitutively-activated Akt molecule was permanently introduced into Myc83 cells, they were dramatically protected from apoptosis induced by PD153035-mediated EGFR signaling block (APPENDIX B, paper 1 and Figure 7 there within). In search of upstream regulators of Akt, our lab discovered that EGF-mediated Akt activation was diminished or abolished by calcium chealation and inhibitors of calcium-calmodulin , suggesting that calcium signaling molecules in Myc83 cells were important modulators of EGF, signaling upstream of Akt (See Figure 5a and 5b, APPENDIX A).

Calcium, a known and important intracellular signaling molecule, exerts many of its signaling properties by interaction with a calcium binding protein called calmodulin (12). Calmodulin, coupled with calcium are able to interact with and activate a variety of kinases termed calcium-calmodulin dependent protein kinases (CaM kinase) (12). CaM kinases are extensively studied in the nervous system where they play a critical role in intra-neuronal signaling, and in muscular tissue where many of their substrates have been identified including cytoseketal proteins such as actin and myosin (12). Their role in mammary epithelium signaling was virtually unknown until a few years ago. Today little is know about the role of CaM kinases in the breast. A few CaM kinases , however, have been discovered to be expressed in both normal and transformed mammary epithelial cells (12, 13). One of these CaM kinases, CaM kinase III

was demonstrated to be selectively active in proliferating cells, with increased specific activity in fresh human breast tumor samples compared to adjacent normal tissue (13). Additionally, CaM kinase III is stimulated by EGF and IGF-1in serum-starved breast cancer cells lines MCF-7 and MDA MB-231 (13). When Myc83 cells were treated with Rottlerin, an inhibitor of CaM kinase III inhibitor and PKC delta, they lost Akt activation, even in the presence of EGF. Specific inhibitors of other CaM kinases had no effect on Akt activation in Myc83 cells. Similarly, The broad spectrum PKC inhibitor, GFcompound, did not disrupt Akt activation in Myc83 cells, eliminating a role for PKC delta in the EGF singaling cascase in Myc83 cells. This data, taken together, suggests that Akt activation and it subsequent survival signals are positively regulated upstream by CaM kinase III

The generation of a mouse mammary carcinoma cell lines derived from MMTV-c-Myc expressing mammary tumors has created an effective *in-vitro* model in which to study the relationship of c-Myc with other mammary-associated proteins involved in the tumorgenic process (14). In our lab and others, transformed cell lines derived from MMTV-*c-myc* mammary tumors are used to study the molecular interactions that contribute to breast cancer (14). In a recent study, a degenerative RT-PCR protein kinase screen to identify kinases involved in mammary development and carcinogenesis resulted in the identification and cloning of a new mouse CaM kinase called PNCK (pregnancy-upregulated nonubiquitous CaM kinase) (15). This CaM kinase, PNCK, with similarites to CaM kinase I, was found to be upregulated during mouse mammary glad development and specifically expressed in the adult mouse during pregnancy (15, 16). Interestingly, PNCK was found to be highest in late pregnancy when alveolar epithelial cells exit the cell cycle and differentiate (16). PNCK was also found to be upregulated in cultured, over-confluent and serum-starved cells compared to actively growing mammary epithelial cells (16). These findings suggest that PNCK expression is inversely related to proliferation in mammary epithelial cells. PNCK, additionally, was found to be upregulated in a number of human breast tumor samples but not in benign tissue, in human breast cancer cell lines and, interestingly, specifically upregulated in mouse mammary tumors resulting from MMTV-driven overexpression of c-Myc, but not from tumors derived from other MMTV-driven transgenic mice (16). These observations suggest that PNCK is also associated with transformation of the mammary epithelium, and possibly in a c-Myc associated manner.

Our lab hypothesized that PNCK expression opposes the Akt activating properties of CaM kinase III in c-Myc expressing mouse mammary carcinoma cells (Myc83). The relationship between PNCK and CaM kinase III in regulation of EGF signaling upstream of Akt in mouse mammary carcinoma cells is currently being investigated in our lab.

Because PNKC is a novel, uncharacterized protein, and since CaM kinase's role in mammary epithelial cell proliferation and transformation is unexplored, we proposed to clone the human PNCK gene and investigate its role in human breast cancer cells.

Revised Aims #1: 1 Year

Hypothesis: We propose that PNCK, the pregnancy upregulated nonubiquitous, CaM kinase has an anti-proliferative or pro-apoptotic effect on mammary epithelial cells during or after the transformation process.

Aim 1: We aim to clone the human PNCK gene and functionally characterize its role in mammary carcinoma cells.

Aim 1a: Cloning of the human PNCK gene.

Since the mouse gene has been cloned, sequenced and mapped to the Xq28 chromosome, we aim to clone the human PNCK by using the mouse sequence to screen human EST databases for homologous matches. Within a few weeks time from beginning the EST database search me came across a recently published nucleotide GeneBank entry U52111, which sequenced through the entire human Xq28 region. Within that entry was a human gene similar to CaM kinase I (17). The DNA sequence of the CaM kinase simailar to human CaM kinase I was 89% homologous to the mouse PNCK gene sequence and 95% conserved at the protein level, with this information, we assumed that the gene published in the Xq28 sequence was the human PNCK gene. Using the human U52111 sequence, we searched the EST data base for possible image clones and found 2 possible matches from a cDNA male human brain library enriched for full length clones. The image clones were purchased and sequenced in total. The human PNCK gene was obtained by PCR of those clones. Currently were are in the process of cloning the human PNCK into a phCMV-HA-tagged expression vector (Gene Therapy Systems, Inc., San Diego, CA) and verifying both the full sequence and proper orientation.

Aim 1b: Transiently and stabally express PNCK, and PNCK mutants in human mammary carcinoma cells compared to empty vector control.

After cloning PNCK into the CMV driven phCMV-HA tagged vector, we will introduce it into mammary carcinoma cells, first transiently and then permanently. PNCK will be ectopicly expressed in human breast cancer cell lines that are negative for the pro-apoptotic kinase DAP-kinase, since DAP kinase might confound our results (18, 19). MCF-7, T47D, MDA-MB-435, MDA-MD-231, MDA-MB-453 and other will be screened for both DAP kinase expression and endogenous PNCK (18). Our lab is currently developing a polyclonal antibody against mouse PNCK. We will transiently introduce PNCK into the human breast cancer cell lines by lipid transfection method with LIPOFECTAMINE 2000 (Invitrogen). If lipid-based methods do not succeed, we will package PNCK into a retrovirus and infect the cell lines with the virus for both transient and stable expression. We will generate both PNCK- HA-tagged and untagged stable cell lines. Simultaneously, catalytically inactive PNCK mutants and mutants deleting the CaM regulatory domain will be generated by site-directed mutagenesis. These will be used in our analysis of PNCK effects on cell cycle and cell death. Stable expression of PNCK will occur with G418 selection which will eliminate all cells not incorporating our PNCK vector with G418 resistance.

Aim 1c: Functionally determine the effect of PNCK expression and PNCK mutants on cell proliferation and apoptosis *in vitro*.

Once PNCK is introduced into the cell we will begin investigating its role on cell proliferation and cell death. We will evaluate the whether PNCK inhibits cell cycle by transfecting PNCK, PNCK kinase dead mutant and vector control into MCF-7, T47D and MDA-MD 231 and after two weeks in selection medium (G418) the drug resistant cells will be stained with crystal violet. FACS analysis will also be performed comparing the percentage of cells in G1, S, G2 and M phases between PNCK and PNCK mutants vs. control. The effect on PNCK and PNCK mutants on apoptosis will be determined by Hoechst stain and evaluation of apoptosis based on nuclear morphology and compared to vector controls. Comparison of apoptotic death between PNCK, PNCK mutants and control will be determined by Annexin staining and PAPR cleavage assays.

If no effect is seen in the PNCK transfected cells, we will determine if the catallytically inactive mutants can protect breast cancer cells from pro-apoptotic stimulus such as INF-γ, FasL, TNFα, radiation.

To further investigate the mode of action of PNCK in mammary epithelial cells, PNCK will be cloned into a FLAG-tagged vector, expressed in breast cancer cells and it intracellular localization will be

visualized by immunostaining with an anti-FLAG antibody. Changes in cellular location of PNCK upon exposure to pro-apoptotic stimuli will also be evaluated with PNCK-FLAG-tagged expression in breast cancer cells

Aim #2: Year 2 We aim to clone the PNCK promoter and determine the fundamental transcriptional regulatory elements controlling PNCK expression.

Aim 2a: Clone the PNCK promoter:

In order to understand the basic transcriptional regulation of the human PNCK, we propose cloning of the PNCK promoter. We aim to do this by cloning the PNCK promoter from a human genomic library by using the PCR-based PromoterFinder DNA Walking kit (Promega). Gene specific primers will be derived from the 5'UTR of the human PNCK gene. The full length promoter will cloned by sequenced. The transcription start site of the human gene will be determined using primer extension analysis with nested primers derived from known DNA sequence.

Aim 2b: Promoter analysis to determine basic transcriptional regulation of the human PNCK gene.

First, sequence analysis of the promoter will be performed to determine the presence of consensus transcription factor binding sites present in the human PNCK promoter sequence. To identify the functional promoter elements involved in PNCK gene regulation, progressive 5' deletion mutants will be constructed based on the location of consensus factor binding sites on the promoter. These promoter mutants as well as the wild-type promoter will be cloned into a luciferase vector, transfected into human breast cancer cells and their relative luciferase activity will be assayed. In addition to the 5' deletion mutations, internal mutation and deletions will be made by PCR based site-directed mutagenisis, based on the results of the 5' deletions. These internal deletions and mutations will be cloned into the luciferase vector for transfection into human breast cancer cells.

Aim 2c: Determine the relationship between c-Myc and PNCK regulation.

Since MMTV-*c-myc* but not MMTV-*ras* mice demonstrated a specific upregulation of PNCK in their mammary tumors, c-Myc may be a direct transcriptional regulator of PNCK (16). Using unique, stable c-Myc expressing mammary epithelial cell line created in our laboratory, (MCF10A-Myc) and a 4-OHT regulatable Myc, Myc-ER (MCF10A-MycER) we can determine if c-Myc activity results in PNCK expression and directly investigate the relationship between c-Myc and PNCK regulation (20).

Conclusions and Relevance of Revised Statement of Work:
The previous stated aims attempt to characterize a novel CaM kinase PNCK both in its function and regulation. The biochemical characterization and evaluation of PNCK will be done by another investigator in the lab simultaneously. The work done cloning, characterizing the function and location of PNCK as well as its regulation will provide me, a Pre-Doctoral candidate with a variety of new experimental and scientific challenges. The insight gained and the maturity developed in the past year working on a project that concluded with both negative and artifactual results will be beneficial to my present project as I am now better able to recognize experimental problems and propose alternatives to get around these problems. I will still be under the guidance and supervision of Dr. Robert Dickson as well as Dr. Tushar B. Deb, a Research Instructor in the lab. This project has the potential to develop my career in both experience and exposure, as the successful completion of his project will result in the completion of my Ph.D. in Tumor Biology and the publication of reasonably two scientific articles at the JBC level or better.

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APPENDIX A
Figures and Supplements

FIGURE 1a: FasL western in Myc83 cells treated with inhibitors of EGFR, PI3K and MEK survival pathways.

FIGURE 1b: Fas western in Myc83 cells treated with inhibitors of EGFR, PI3K and MEK survival pathways.

FIGURE 2a: Fas-Fc decoy receptor does not protect Myc83 cells from apoptosis induced by inhibition of EGFR

FIGURE 2b: Fas-Fc decoy receptor inhibits rFasL induced apoptosis in Myc83.

SUPPLEMENT 1: "Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape" Nicholas P. Restifo (Nature 2000)

SUPPLEMENT 2: "Constitutive Expression of FasL in Thymocytes" TECHNICAL COMMENTS (Science 1998)

FIGURE 3: Comparative westerns: FasL clone 33 versus FasL clone N-20

FIGURE 4: MryAkt-Myc83 cells do not induce FasL under conditions which cause apoptosis in Myc83 cells

FIGURE 5a: Calcium chelation with BAPT-AM eliminates EGF induced Akt activation in Myc83 cells

FIGURE 5b: Inhibitors of calmodulin (W-7) but not its control, inhibit EGF-induced Akt activation in Myc83 cells

FIGURE 6: Rottlerrin, an inhibitor of CaM kinase III diminishes EGF-induced Akt activation in Myc83 cells. Inhibitors of other CaM kinases have no effect.

Figure 1a

1a. Inhibition of survival signals downstream of EGFR induces FasL protein expression in Myc83 cells. The EGFR tyrosine kinase inhibitor PD1530305, the P13K inhibitor LY294002, and the MEK/Erk inhibitor U0126 all induce FasL expression after 48 hours of treatment.

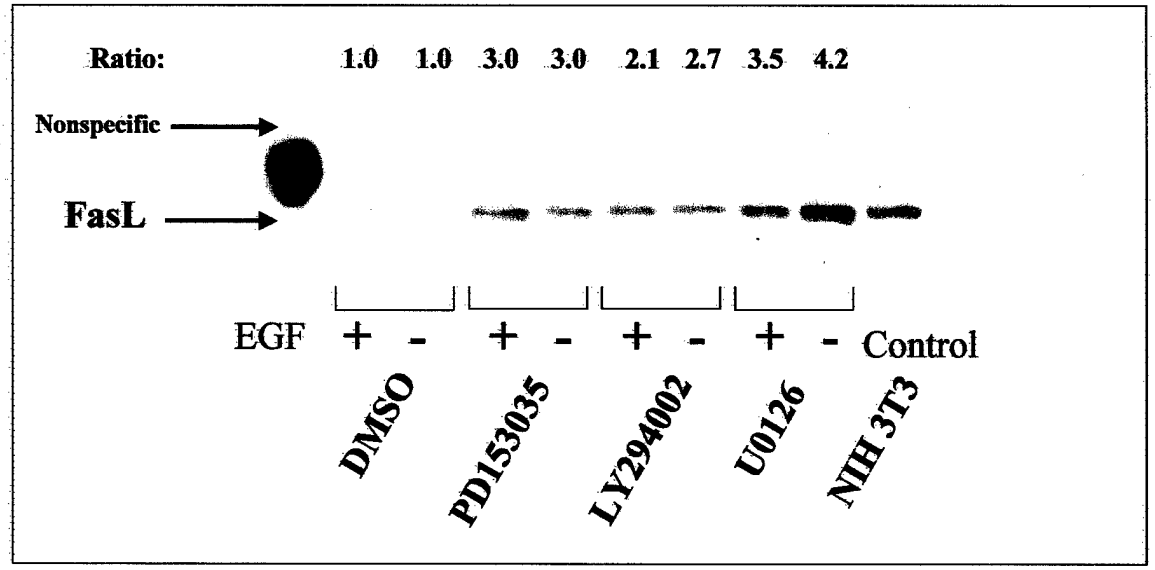


Figure 1b

1b. Fas protein is upregulated in Myc83 cells treated with the EGFR inhibitor PD153035, and the MEK/Erk inhibitor U0126.

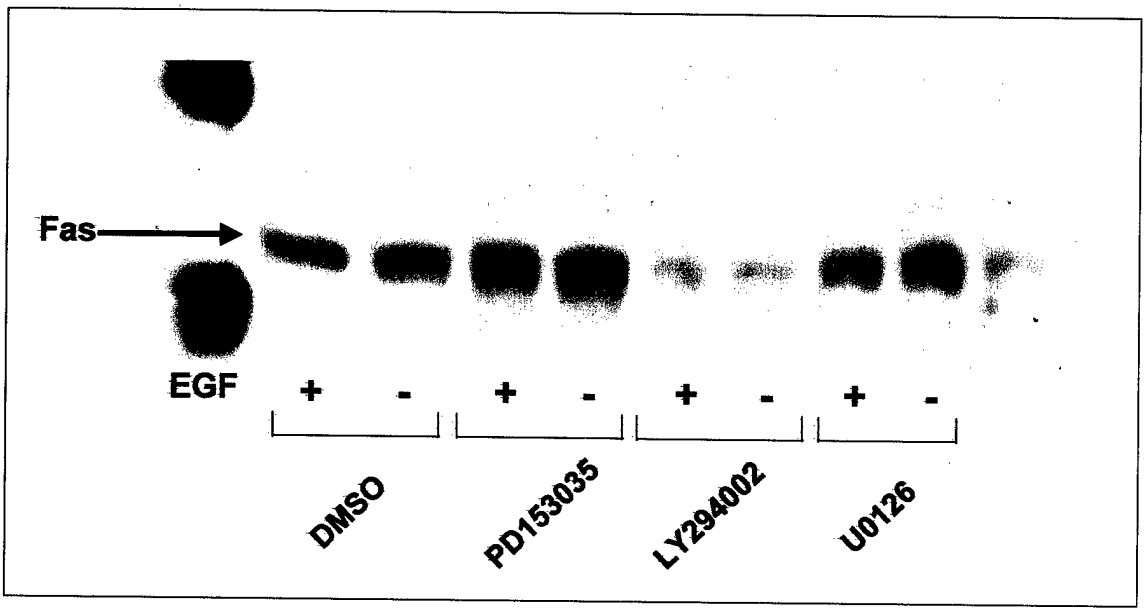


Figure 2a

2a: Pretreatment with a Fas-Fc decoy receptor did not protect Myc83 from apoptosis induced by EFGR blockade.

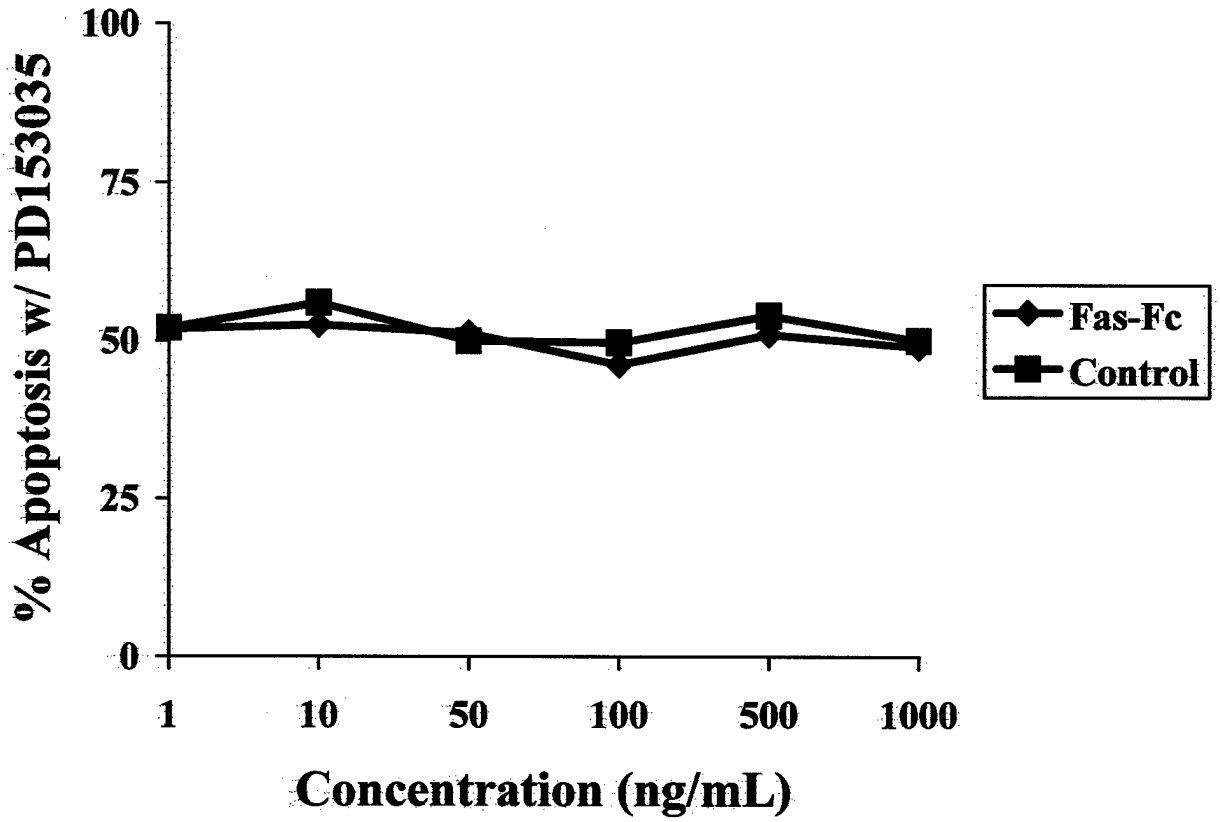
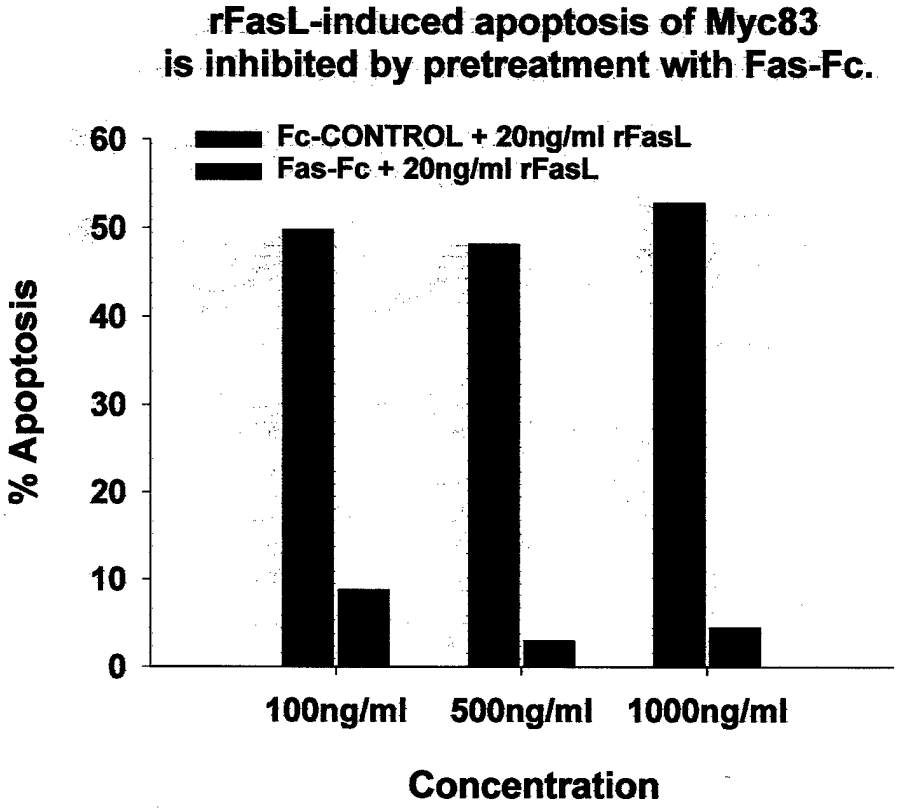


Figure 2b

2b:


rFasL-induced apoptosis of Myc83
is inhibited by pretreatment with Fas-Fc.
Fc-CONTROL + 20ng/ml rFasL
Fas-Fc + 20ng/ml rFasL
% Apoptosis
60
50
40
30
20
10
0
100ng/ml
500ng/ml
1000ng/ml
Concentration

Based on early studies, it was hypothesized that expression of Fas ligand (FasL) by tumor cells enabled them to counterattack the immune system, and that transplant rejection could be prevented by expressing FasL on transplanted organs. More recent studies have indicated that the notion of FasL as a mediator of immune privilege needed to be reconsidered, and taught a valuable lesson about making broad conclusions based on small amounts of data.

Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape

NICHOLAS P. RESTIFO

The history of science is full of ideas that are at once so elegant and so obvious that they take root in our imaginations, despite relatively weak support by available data. One such idea now in the tumor immunology community is that tumor cells use a molecule called Fas ligand (FasL) to counter-attack the immune system[1]. Despite substantial evidence to the contrary, this molecule is thought by some to induce the death and elimination of T lymphocytes that enter the tumor bed, thereby granting the tumor immune-privileged status. But this idea is based on inference: all well-controlled experiments in which FasL expression is induced in a tumor or tissue, either through use of transgenic mice or by transfection or transduction of transplanted cells, have shown that the tissue is rapidly rejected, without evidence of immune privilege.

The original idea was logical. FasL expressed on tissues would engage the Fas receptor expressed on the surfaces of immune cells, causing them to undergo programmed cell death (Fig. 1). The idea that FasL (also known as CD95 ligand or APO-1 ligand) could help tumors counter-attack the immune system has its origins in the field of transplantation. However, several early papers containing evidence initially thought to support the hypothesis that FasL could grant immune-privileged status have now been withdrawn or refuted.

The concept was brought to popular attention in a *News and Views* article published in *Nature* in October 1995 (ref. 2). The author summarized findings demonstrating that Sertoli cells expressing FasL could be transplanted into allogeneic mice and concluded, based on the data, that the interaction of Fas and its ligand was at the heart of immune privilege[3]. Another group then reported that transplanted tissue could be protected from rejection if one simply surrounded tissue (in this case the insulin-producing Islet cells of the pancreas) with myoblasts expressing FasL (ref. 4). Fas–FasL interactions were also reported to be fundamental to immune privilege in the eye[5]. The implications of these findings were great: Graft rejection could be prevented if cells or organs were transfected with FasL before transplantation.

Shortly after these initial publications, two sets of data were published that had a substantial effect on the tumor immunology community, catalyzing a flurry of research activity based on these findings. The first described FasL expression by colon tumor cells, claiming that this induced cell death of Jurkat cells (a human T-cell leukemia line)[6]. The second described a similar set of results using melanoma[7]. The latter demonstrated a remarkable consistency of FasL expression in tumor cell samples: All of the 10 melanomas tested expressed FasL. Moreover, it showed that this FasL was functional. The authors also claimed that infiltrating T cells could only be found proximal to FasL-positive lesions of human metastatic melanoma, a finding at odds with many other observations of tumor-infiltrating lymphocytes. Furthermore, the authors reported that the highly virulent mouse melanoma cell line, B16, was FasL-positive. The authors asserted that FasL expression by B16 caused rapid tumor formation.

After those reports, it seemed that the evidence on the function of FasL in transplantation, autoimmunity and tumor escape was clear and compelling. But some other experimental data did not fit the newly established paradigm of FasL as the enforcer of immune privilege. When Allison *et al.* used transgenic mice expressing FasL on their islet β cells for transplantation, they found that rather than being the solution to the transplantation immunologist's rejection problem, expression of FasL caused a more rapid rejection of islet cells accompanied by a "granulocytic infiltration" (ref. 8). Kang *et al.* used an entirely different approach to test the same question, using adenoviruses to confer FasL expression on islet cells. They also found accelerated rejection accompanied by "massive neutrophilic infiltrates" (ref. 9). Similar results were obtained using other cells[10,11]. Over the two years that followed, Seino *et al.* would publish many papers testing a possible use for FasL in transplantation, but their data refuted the idea that conferring FasL expression on transplants would be therapeutically useful as originally described[6,12–15]. Instead, ectopic FasL expression caused rapid rejection and profound inflammation with abscess formation.

In vitro and *in vivo* experiments with tumor cells also contradicted the original hypothesis that FasL mediated an immune counter-attack. Some investigators claimed that they could not find FasL on the surfaces of melanoma cells[16,17], and when they transfected tumor cells with the gene encoding FasL, they did not detect 'tumor escape'. Instead, there was rapid tumor rejection in many experimental tumor systems (including the B16 melanoma)[10,17]. As with other FasL-based transplantation experiments, rejected tumors sites were infiltrated with granulocytes that coalesced into abscesses. Soluble FasL can, in part, abrogate the inflammatory effects of membrane-bound FasL (ref. 18).

So why did FasL trigger inflammation? A definitive answer to this question is unknown. However, it is clear that Fas signaling activates a caspase cascade. One consequence of this cascade is the activation of interleukin-1β-converting enzyme (ICE), also known as caspase 1 (ref. 19). As its name indicates, ICE is capable of cleaving interleukin-1β from its inactive form into its active form[20]. Once activated, interleukin-1β is a potent pro-inflammatory cytokine. ICE also cleaves and activates IL-18: the disabling of ICE is a strategy used by poxviruses to evade immune destruction[21].

Although some early authors sought to retract their earlier statements, others 'stuck to their guns'. It was important for trans-

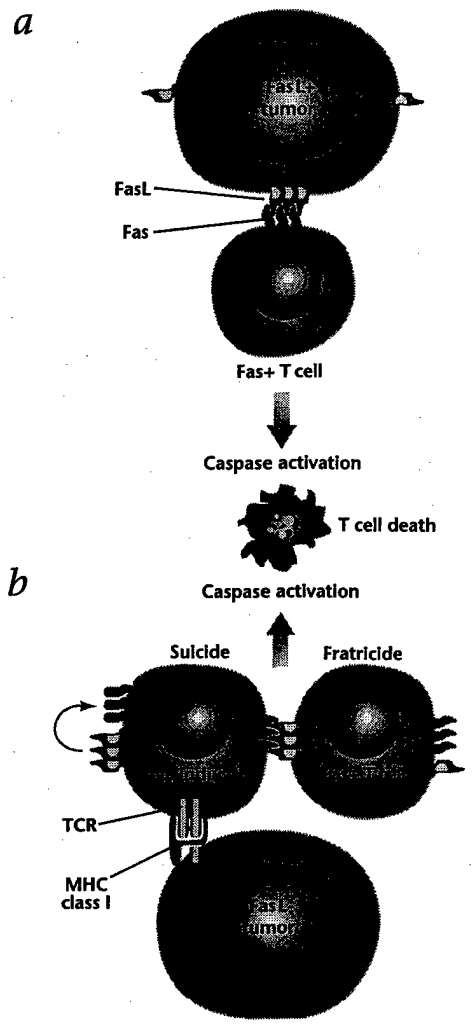


Fig. 1 FasL-induced T cell death. ***a***, Early experiments indicated that FasL, expressed on tumor cells, interacts with its receptor, Fas, which is expressed on invading T cells. This interaction would trigger T-cell death, through caspase activation, and grant the tumor immune privilege. ***b***, Recent studies have shown that FasL is expressed by T lymphocytes after tumor recognition and T cell activation. T cells then kill themselves ('suicide') and each other ('fratricide') through the same caspase-based mechanism.

plant immunologists to correct their original mistakes, as transplants engineered to express FasL could prove to be disastrous, leading to rapid rejection and abscess formation rather than increased tissue engraftment. Vaux, who wrote one of the earliest and clearest descriptions of FasL as the 'enforcer' of immune privilege, took the unusual step of retracting his *News and Views* piece after his own lab found inflammation, not immunosuppression[2].

The continued proliferation of erroneous ideas about FasL may have been due in part to technical problems hindering early research. A principal problem involved the antibodies used in the early reports. A polyclonal antibody against human FasL (C-20), produced by Santa Cruz Biotechnologies, was not highly specific[22], leading to the publication of false-positive results on a least a half-dozen different occasions by the time its cross-reactivity was discovered in 1998, and in several reports by others since then[23]. Compounding the problem may be the fact that many other polysera were generated in a similar way[24], but were not tested in 1998 report[22]. An underlying and ongoing problem is the use of polysera made by injecting animals with peptides. Although the generation of polyclonal antibodies is straightforward, a complete characterization of antibody specificity is difficult and fraught with pitfalls. Another widely used reagent, a mouse monoclonal antibody (clone 33) from Transduction Laboratories, was also reported to be nonspecific for FasL (ref. 25).

Other possible confounding variables include the use of non-intron-spanning PCR primers without proper controls[7,26], contamination of fresh tumor samples with lymphocytes (which can express large amounts of FasL) and problems with functional assays. For example, several studies involved T-cell targets that were themselves capable of expressing FasL, such as Jurkat cells. When target cell death was found and blocked with antibodies against FasL, researchers were not always able to verify that the cell death was induced by FasL expression on tumor cells and not by induced expression of FasL on T cells[26]. Thus, the devil really was in the details.

One consequence of these misunderstandings, for tumor immunology, was that researchers have been side-tracked in determining the true biological function of Fas and FasL as mediators of cytotoxicity[27] and as central mediators of activation-induced cell death[28]. Thus, it is the case that Fas-FasL interactions can cause T cell death and this death is important in the induction of tolerance, immune homeostasis and lymphocyte effector functions. Indeed, it has been confirmed that melanoma-specific T lymphocytes undergo apoptotic death after the major-histocompatibility-complex-restricted recognition of tumor cells, and T-cell death can be blocked by the addition of a specific antibodies against Fas (ref. 29). However, contrary to the prevailing view that tumor cells cause the death of anti-tumor T cells by expressing FasL, it is now apparent that in most cases, FasL is expressed by T lymphocytes upon activation after tumor cell recognition, causing them to kill themselves ('suicide') and each other ('fratricide') (Fig. 1)[16,29,30]. When FasL is expressed ectopically with the goal of inducing the death of T lymphocytes, researchers must consider the resultant caspase cascade and the potential for the consequent activation of an innate immune response.

Hindsight is always perfect, and it is now possible to view initial mistakes and contradictory data regarding the function of FasL in a new light. A tantalizing new idea, featured prominently in the literature, can rapidly spread through the scientific community. Once a widely believed hypothesis is re-evaluated, 'cool' negative results may not receive the same attention as 'hot' positive results. Furthermore, corrections and retractions, as in politics, are often hidden in less-than-obvious places, such as during the question-and-answer periods of lectures, or in abstracts or posters at scientific meetings. It may be true that science is a self-correcting enterprise, and theories come and go, but even in this age of rapid communication, the progress of truth can be glacially slow. Scientific researchers and journal editors must be ever-wary of elegant and intuitively obvious ideas that are only weakly supported by available data.

Acknowledgments

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Constitutive Expression of FasL in Thyrocytes

C. Giordano *et al.* (*1*) reported evidence that the ligand for Fas antigen (FasL) is constitutively expressed on thyroid follicular cells from both normal and Hashimoto's thyroiditis (HT) tissue, and that normal thyrocytes express Fas antigen only after induction with interleukin-1β (IL-1β). Giordano *et al.* conclude that their results suggested a possible mechanism for thyrocyte cytotoxicity in autoimmune thyroiditis. The absence of Fas antigen expression on the surface of normal thyrocytes has been supported by one study (*2*); however, other studies have found Fas expression by normal thyrocytes both in situ (*3*) and in primary cultured cells (*4*, *5*). The findings by Giordano *et al.* raised the possibility of the expression of both Fas antigen and FasL on normal thyrocytes. To clarify this issue, we examined the expression of both FasL and Fas antigen mRNA in primary cultured thyrocytes with the use of reverse-transcriptase polymerase chain reaction (RT-PCR) (*6*) and ribonuclease protection (*7*) techniques. Neither assay, performed on RNA isolated from normal human thyrocytes, demonstrated mRNA for FasL (Fig. 1, A and B). To assure that this result was not unique to this sample, RNA samples from five different normal and Graves's diseased thyrocytes were screened by ribonuclease protection assay; these also did not show mRNA for FasL (Fig. 1C). In contrast, Fas antigen mRNA was detected in all five specimens (Fig. 1C) and has also been detected by RT-PCR (*5*). Treating the thyroid cells with TSH, IL-1β, or γINF for up to 48 hours (before harvest and RNA isolation) also did not induce the expression of FasL mRNA or alter the expression of Fas antigen mRNA.

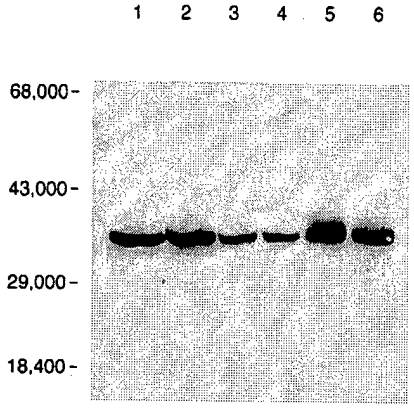

Fig. 2. Protein immunoblot analysis of cell lysates probed with a monoclonal antibody for FasL (clone 33, Transduction Technologies). Cell lysates correspond to the following lane numbers: (1) unstimulated Jurkat cells; (2) activated Jurkat cells; (3) thyroid goiter, cultured with TSH; (4) thyroid goiter, cultured without TSH; (5) normal thyroid cultured with TSH; and (6) normal thyroid cultured without TSH. A heavy band is visible in all samples at roughly 37 kD, the reported size of FasL. Presence of the bands in all samples, including resting Jurkat samples that were from cells devoid of FasL mRNA, is inconsistent with the data obtained from the immunohistochemical staining, RT-PCR, and nuclease protection assays.

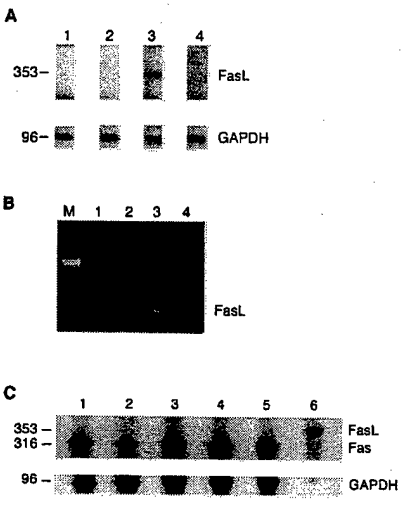

Fig. 1. (**A**) Ribonuclease protection assay for FasL mRNA. Protected fragment of FasL mRNA yielded a 353-bp band, while a 96-bp band from a protected fragment of GAPDH mRNA was used to standardize RNA concentrations. RNA from two different individual's primary cultured thyrocytes (lanes 1 and 2) showed no message for FasL. An equal amount of RNA isolated from Jurkat cells stimulated with PMA (lane 3) demonstrated FasL mRNA and served as a positive control. RNA isolated from untreated Jurkat cells (lane 4) also was negative for FasL mRNA. (**B**) RT-PCR assay for FasL mRNA. Primers were designed to yield a 663-bp product corresponding to bp 796 to 1459 of Fasl mRNA. RNA from normal thyrocytes that were either TSH-deprived (lane 1) or TSH-supplemented (100 mU/ml) (lane 2) showed no FasL mRNA. RNA from Jurkat cells stimulated with PMA served as a positive control (lane 3), while no message was observed in RNA from untreated Jurkat cells (lane 4). RT-negative amplifications showed no product in the positive control (data not shown). (**C**) Ribonuclease protection assay for FasL and Fas mRNA. RNA from two additional normal (lanes 1 and 2) and three Graves (lanes 3 to 5) primary cultured thyrocytes was analysed. Although message for FasL was not detected in any of the thyrocyte samples, a 316-bp protected fragment of Fas mRNA was detected in all of the thyrocyte RNA preparations. A small amount of RNA from peripheral blood lymphocytes stimulated with PMA (lane 6) served as a positive control for FasL mRNA.

We also examined the expression of FasL protein in thyroid follicular cells with the use of immunohistochemical staining (*8*) and Western blotting (*9*) techniques similar to those employed by Giordano *et al.* (*1*). Immunostaining of normal thyrocytes with a polyclonal, rabbit antibody to FasL did not detect FasL protein (*10*), but, consistent with the other studies, these cells demonstrated significant amounts of Fas antigen staining (*2*–*4*). Unexpectedly, a protein immunoblot of thyroid cell lysates performed with the mouse monoclonal antibody used by Giordano *et al.* (clone 33, Transduction Laboratories, Lexington, Kentucky) yielded an intense band at the appropriate size for FasL (Fig. 2). However, there was no difference in intensity of this band in lysates of PMA stimulated as opposed to untreated Jurkat cells, and this finding did not correlate with the changes in mRNA concentration observed in these cells (Fig. 1, A and B). This result brings into question the specificity of the clone 33 antibody for FasL.

Although there may be differences in the tissues examined by ourselves and Giordano *et al.*, we have not observed the expression of mRNA for FasL in primary cultured thyrocytes from over 20 normal and thyroiditis tissue samples, unless there was also evidence of mRNA for rearranged immunoglobulin genes (*10*). The latter result suggests that, in those situations, the message for FasL came from lymphocyte contamination of the thyroid cells. More importantly, FasL-induced programmed cell death in thyroiditis is questioned by our recent finding that the Fas pathway in thyroid follicular cells is blocked by a labile protein inhibitor (*5*). It has also been found that the in vitro induction of the Fas pathway with soluble ligand or antibody may be less efficient than that achieved by cytotoxic T cells (*11*). Together, these considerations make it difficult to predict the relative importance of Fas-mediated apoptosis in thyroiditis. We hope that the findings presented in this comment will promote the research and discussion necessary to clarify the potential role of the Fas pathway in the pathogenesis of thyroiditis.

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6. For RT-PCR, RNA was isolated with the use of Tri Reagent (Molecular Research Center, Cincinnati, OH). One microgram of total RNA was used in a first-strand cDNA synthesis with 100 ng of oligo(dT)18 and amplified by PCR. PCR was performed at 94°C for 2 min followed by 30 to 34 cycles of amplification. Each cycle consisted of 35 s of denaturation at 94°C, 35 s of annealing at 58°C, and 45 s for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow re-annealing of the amplified products. PCR products were then size-fractionated through a 2% agarose gel and the bands visualized with the use of ethidium bromide. A pair of primers was used to amplify human β-actin as a control. Primer sequences:
FasL:
forward primer: 5′-ACAACCTGCCCCTGAGCC-3′;
reverse primer: 5′-AGTCTTCCTTTTCCATCCC-3′;
β-actin:
forward primer: 5′-CACGGCATTGTAACCAACTG-3′;
reverse primer: 5′-TCTCAGCTGTGGTGGTGAAG-3′.
7. For ribonuclease (RNase) protection, the RiboQuant MultiProbe RNase Protection Assay System (Pharmingen, San Diego, CA) was used for the detection and quantitation of specific mRNA species. ^{32}P-labeled antisense RNA probes were prepared with the use of the Human Apoptosis hAPO-3 Template Set (Pharmingen), which included Fas, FasL, and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Probes were hybridized with 10 μg of RNA from treated thyrocytes, Jurkat cells, and peripheral blood lymphocytes. After hybridization, samples were subjected to RNase treatment followed by purification of RNase-protected probes. Protected probes were resolved on a 5% denaturing polyacrylamide gel.
8. Thyroid cells were grown on Falcon chamber slides (Becton Dickinson, Franklin Lanes, NJ) or on glass coverslips. For immunostaining, slides were washed twice with PBS, then fixed with methanol for 5 min at 4°C, briefly air-dried, placed into PBS and blocked with 5% normal goat serum. Slides were incubated with 1.0 μg/ml affinity purified rabbit antibody to Fas (C-20, Santa Cruz Biotechnology), antibody to FasL (Q-20, Santa Cruz Biotechnology), or rabbit antithyroglobulin (Dako Corp., Carpinteria, CA). After washing with PBS, slides were incubated with biotinylated Abs specific for rabbit IgG, followed by detection using an avidin-biotin complex detection kit with glucose oxidase substrate (Vectastain ABC-GO kit, Vector Labs., Burlingame, CA). Slides were briefly counterstained with eosin and mounted with permount (Fisher Scientific, Fair Lawn, NJ).
9. Thyrocytes were scraped from tissue culture dishes and collected by centrifugation at 200 g for 10 min. Cell pellets were lysed in cold lysis buffer (0.5% Triton X-100 in 50 mM tris, pH 7.6, 300 mM NaCl) containing protease inhibitors for 30 min on ice, then centrifuged at 15,000*g* for 20 min, and the Triton-soluble protein fraction was collected. Total protein concentrations were determined using BCA protein assay (Pierce Chemicals, Rockford, IL). As a positive control for expression of FasL, protein lysates were also prepared from Jurkat cells that were activated using PMA and ionomycin for 4 hours. Equal amounts of cell protein lysates were mixed with 2X sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol in 0.125 M tris, pH 6.8), samples were boiled for 3 min, then separated by electrophoresis on a 12.5% SDS-polyacrylamide gel. Proteins were then electrophoretically transferred to nitrocellulose. Blots were first blocked in 5% milk in PBS with 0.02% sodium azide (PBS-A) for 2 hours at 25°C. Blots were incubated overnight at 4°C with mouse monoclonal anti-FasL (Clone 33, Transduction Laboratories, Lexington, KY). After washing with PBS with 0.02% sodium azide and 0.05% Tween-20 (PTA), blots were then incubated with anti-mouse IgG alkaline phosphatase conjugated antibody (both from Jackson ImmunoResearch Labs., West Grove, PA) diluted 1:2500 in 5% milk/PBS-A for 1 to 2 hours at 25°C. Blots were again washed and developed with a BCIP/NBT substrate.
10. T. A. Stokes *et al.*, data not shown.
11. M. Tanaka *et al.*, *Nature Med.* **4**, 31 (1998).

10 December 1997; accepted 24 February 1998

Giordano *et al.* describe the constitutive expression of Fas ligand (CD95L) by thyrocytes that they isolated from the glands of patients with Hashimoto's thyroiditis or nontoxic goiter (*1*). On the basis of this unexpected result, Giordano *et al.* propose that the concommitant expression of Fas and its ligand induces programmed cell death of thyrocytes and that this might be a major pathological mechanism underlying many forms of hypothyroidism. To detect FasL expression, Giordano *et al.* used polymerase chain reaction (PCR), immunohistochemical stainings, and FACS analysis. For the latter experiments, they used two commercially available antibodies against FasL, C-20 and mAb33. C-20 (Santa Cruz Biotechnology, Santa Cruz, California) is a rabbit polyclonal IgG antibody against an extracellular FasL epitope corresponding to amino acid residues 260 to 279, and it is recommended for use in protein immunoblots and immunohistochemistry. Monoclonal antibody mAb33 (Transduction Laboratories, Lexington, Kentucky), is an IgG1 monoclonal antibody against the extracellular part (216-277) of human FasL. It is made for the study of human FasL by protein immunoblots, immunoprecipitation, and immunofluorescence.

With the use of monoclonal antibody 33 (mAb33), we analyzed FasL expression with protein immunoblots in a panel of human tumor cell lines covering different B cell, monocyte, and T cell lines. Unexpectedly, we found that all these cell lines express FasL, as shown by a single band on the blot at about 37 kD. To confirm these results, we tested these cell lysates with a FasL-specific rabbit polyclonal antiserum PE62 against an extracellular peptide of FasL (*2*). In contrast to the results obtained with mAb33, none of these cell lines now showed a FasL-specific signal (Fig. 1A).

We therefore examined further the FasL specificity of mAb33. We transiently transfected human 293T embryonic kidney carcinoma cells with a FasL expression vector

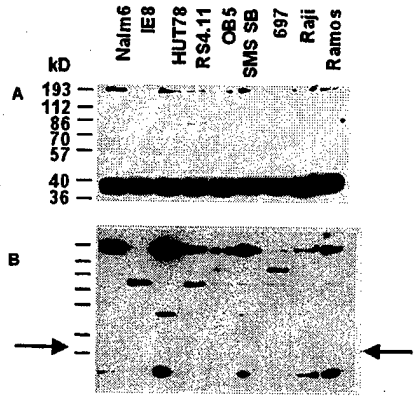

Fig. 1. FasL expression in cell lines. Total cell lysates prepared from 16 different tumor cell lines (eight are not shown) were tested by Western blotting for FasL expression with the use of either mAb33 (**A**) or the rabbit polyclonal antibody PE62 (**B**). Arrows indicate the position of the signal obtained with mAb33. Molecular weight markers were run in parallel as indicated. Cells were lysed in PBS containing 1% NP40 and a mixture of protease inhibitors. Total cell lysates were separated on a 12% SDS-PAGE, blotted against nitrocellulose filter, and analyzed with the antibodies as indicated with the use of HRPO-coupled goat antibody to mouse, rat, or rabbit IgG (Southern Biotechnology Associates, Birmingham, Alabama) and enhanced chemoluminescence (Pierce).

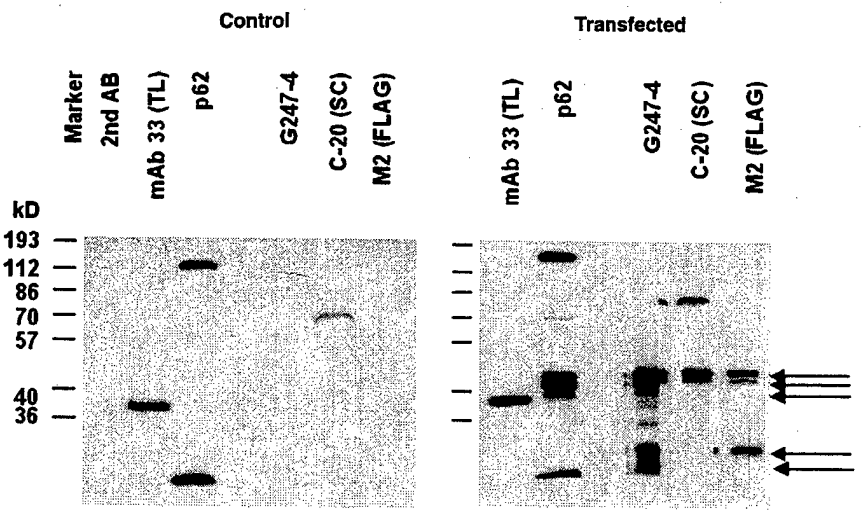

Fig. 2. Analysis of FasL expression in 293 T cells transfected with a FLAG-FasL expression vector. (**Left**) analysis of untransfected cells; (**right**) analysis of transfected cells. Total cell lysates were incubated with antibodies as indicated and analyzed with a protein immunoblot. Arrows show FasL-specific signals. Signal detected by mAb33 is not found with any of the other FasL-specific antibodies.

that encodes human FasL that has an NH_2-terminal FLAG tag, and we performed a protein immunoblot, with the use of the FasL-specific antibodies mAb33, C-20, G247-4 (Pharmingen), and P62 (*2*), as well as M2 (Eastman Kodak, New Haven, Connecticut), which is specific for the NH_2-terminal FLAG tag (Fig. 1B).

With the use of mAbs C247-4 and M2, the FasL-transfected 293T cells showed FasL-specific signals that were absent in the untransfected control cells. Although the polyclonal rabbit antibodies C-20 and PE62 showed some background staining in untransfected 293T cells, they also revealed CD95L-specific signals in the lysates of the transfectants, as would be expected from earlier reports (*2–4*). In contrast, mAb33 detected a 37-kD signal (similar to the band in Fig. 1A) in both transfected and untransfected cells. To further characterize the specificity of mAb33, we immunoprecipitated FLAG-tagged FasL from lysates of transfected 293T cells with the use of the FLAG-specific antibody M2. The immunoprecipitates were tested by protein immunoblotting with G247-4 and mAb33. FasL expression was detected with G247-4, but not with mAb33. However, mA33 (but not G247-4) produced a strong 37-kD signal in the supernatant of the immunoprecipitate (Fig. 1C).

Thus, C-20, PE62, and G247-4—but not mAb33—seem to be suited for the analysis of CD95L expression by protein immunoblotting. However, mAb33 does not stain human FasL, but a different protein expressed in many cell types. It is therefore questionable whether the signals shown in figure 4 of the report by Giordano *et al.* correspond to FasL expressed by thyroid cells.

With the use of 293T cells transfected with FLAG-tagged FasL, we then analysed several antibodies (NOK-1, NOK-2 (*5*), and G247-4 (PharmIngen, San Diego, California), mAb33, C-20, and MIKE 2 (Alexis, San Diego, California) for FasL specific staining with the use of flow cytometry and immunoflorescence. Untransfected cells served as specificity controls. With the use of a fluorescent activated cell sorter (FACS), only NOK-1, NOK-2, and MIKE-2 detected FasL expressed on the 293T cell transfectants, whereas C-20, mAb33, and G247-4 did not show any specific signals (*6*). G247-4 is known to work only in Western blots, but C-20 was also reported by Giordano *et al.* to stain FasL-expressing thyrocytes with the use of flow cytometrical and immunohistochemical analyses. In the immunoflurescence studies, FasL-specific signals were easily detected with NOK-1, G247-4, and with the FLAG-specific antibody M2. The peptide-specific rabbit polyclonal antibody C-20, however, gave a high background staining already with untransfected cells. FasL transfectants showed stronger signals, but also a similar background staining similar to the controls. Both stainings were not seen when the blocking peptide was added (*6*).

On the basis of these results, we conclude that C-20 is suited for analyzing CD95L expression by protein immunoblotting, but not by flow cytrometry, immunofluorescence, or immunohistochemistry. Because C-20 detects in such blots a signal of about 65 kD, which does not correspond to FasL (Fig. 1B), it may also stain by immunofluorescence antigens unrelated to FasL that may bear epitopes similar to the peptide used to generate the antibody. Flow cytometrical experiments using C-20 may also reveal cells expressing such epitopes that may be absent in 293 T cells but not in thyroid cells. We therefore cannot recommend the application of C-20 in flow cytrometrical and immunofluorescence studies designed to show FasL expression.

In comparison to the other FasL-specific antibodies, mAb33 detects a different intracellular protein that seems to be expressed ubiquitously. Previous studies that used this antibody should be interpreted with caution. In conclusion, our studies describe a panel of antibodies that are specific for FasL and that may be used either in flow cytrometrical, immunofluorescence, or Western blot analysis. They also show that the two antibodies C-20 and mAb33 may bear additional specificities or might not be specific for FasL. Because both antibodies are commercially available, they seem to be used frequently by many investigators. We suggest that results from such studies (*1*, *7*) should be reinterpreted keeping in mind the specificity of these antibodies, or repeated with the use of reagents that are known to be specific for FasL.

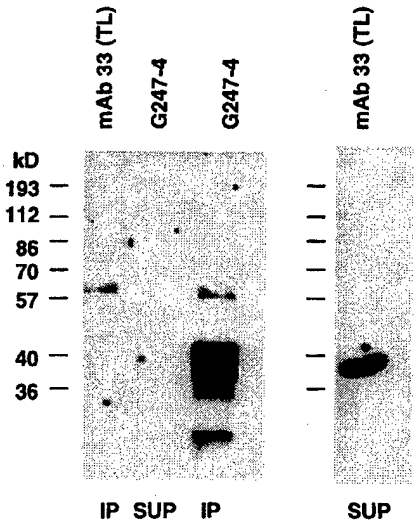

Fig. 3. Total cell lysates from FLAG-FasL–transfected 293 T cells were incubated with the FLAG-specific mAb M2 coupled to agarose beads (Kodak). FasL was immunoprecipitated and subjected to Western blotting. Nitrocellulose filters were developed with G247-4, or with mAb33 and HRPO-coupled goat–anti-mouse IgG. Mab33 detects a 37-kD signal present in the supernatant, but not immunoprecipitated FasL.

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6. P. Fiedler and H. Eibel, data not shown.
7. G. A. Niehans *et al.*, *Cancer Res.* **57**, 1007 (1997).
8. We thank J. Tschopp and M. Hahne for providing the rabbit polyclonal antibody PE62 and for helpful discussions. Part of the work was supported by grant Nr. Ei235/4-1 to H.E.

20 January 1998; accepted 24 February 1998

Response: We appreciate the comments by Stokes *et al.* and Fiedler *et al.* about our earlier report (*1*). With regard to detecting FasL in primary thyrocyte cultures with RNA protection assay and PCR, such detection would be difficult if the thyrocytes were not freshly excised and immediately analyzed, because FasL expression is labile ex vivo (mRNA or protein).

Concerning Fas expression, Stokes *et al.* used thyrocytes from controlateral lobes of thyroid cancers as their "normal" controls (*2*). We also observed variable Fas expression in uninvolved thyrocytes from thyroids with cancer, but we did not use such samples for normal controls. We obtained more normal samples from thyroid sections from patients undergoing laringectomy for laringeal cancer. These thyrocytes consistently expressed very low amounts of Fas, similar to thyrocytes from patients with nontoxic goiters (NTG), which we used in our report (*1*).

Prompted by concerns raised about the specificity of the polyclonal antibody to FasL C20 (Santa Cruz, Biotechnology, Santa Cruz, California) and mAb 33 (Transduction Laboratories, Lexington, Kentucky) (see below), we repeated an immunohistochemical study with the NOK-2 antibody (PharMingen, San Diego, California) on thyroid sections from laringectomy patients (above). Most thyrocytes from normal thyroids showed detectable FasL expression (*3*) (Fig. 1). In situ hybridization would further address this issue. Moreover, FACS analysis

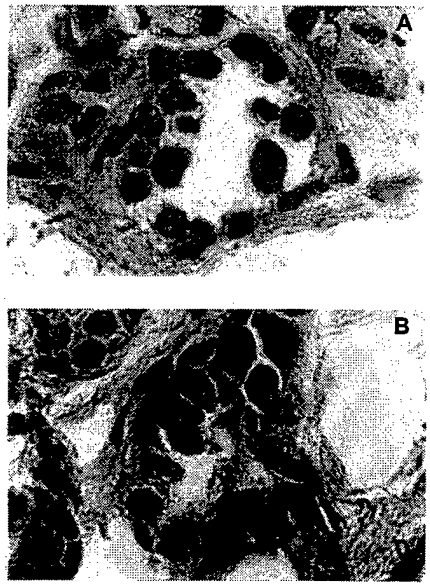

Fig. 1. Immunoistolocalization of FasL in thyroid tissue from a laringectomized patient. (**A**) control mAb, (**B**) NOK-2 mAb.

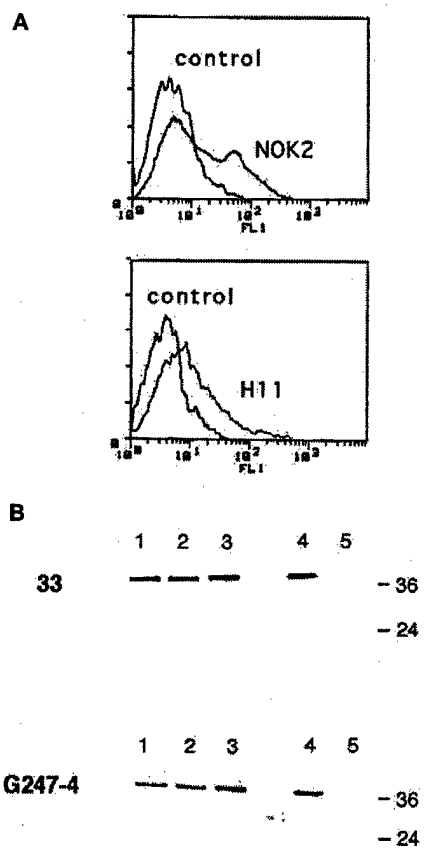

Fig. 2. (**A**) Surface FasL expression of ex vivo NTG thyrocytes detected by NOK-2 and H11 antibodies. Species/isotype-matched antibodies were used as controls. (**B**) Protein immunoblot analysis of FasL expression in thyrocytes as detected by mAbs 33 and G247-4. Lane 1: thyroid tissue from a NTG patient. Lane 2: thyroid tissue from a laringectomized patient. Lane 3: thyrocytes from a laringectomized patient, immunodepleted from CD45+ cells. Lane 4: 8 hours PMA-activated Jurkat J77 cells. Lane 5: untreated Jurkat J77 cells.

of ex vivo thyrocytes from NTG patients confirmed the constitutive expression of FasL on most thyrocytes, with the use of both the NOK-2 antibody and the H11 (Alexis, San Diego, California) antibody (Fig. 2A).

Finally, (i) thyroid tissue from patients with a laringectomy or NTG and (ii) hematopoietic cell-depleted thyrocytes from patients with NTG expressed FasL, as detected by Western blot analysis with 33 mAb (Transduction Laboratories) or with G247-4 mAb (PharMingen) (Fig. 2B). We detect a single band in our blots, similar to some investigators (4), while others detect multiple bands, which might represent glycosylated and unglycosylated forms (5).

Although we agree that C20 antibody may give a relatively high background signal as compared with other commercially available reagents, these new data support our earlier conclusion that normal thyrocytes express substantial amounts of FasL in vivo.

Fiedler *et al.* conclude that mAb 33 recognizes a protein different from FasL. We performed similar experiments with the use of three different cellular systems: (i) 293T cells transiently transfected with human FasL, (ii) COS-7 cells transiently transfected with human FasL (6), and (iii) NIH 3T3 stably transfected with murine FasL (7). To detect the expression of FasL by FACS analysis, we used NOK-1 mAb (PharMingen), C20, G247-4 mAb, and 33 mAb. Species and isotype-matched antibodies were used as control primary reagents. All of these antibodies gave a specific staining only in transfected cells (Fig. 3; only data on NIH 3T3 are shown), although with different distributions.

It is not clear why Fiedler *et al.* did not detect FasL expression by FACS analysis (with the use of the 33, C20, and G247-4 antibodies), or why the FLAG-FasL immunoprecipitate reacts with G247-4, but not with the mAb 33. We used a human FasL construct; they used a human FasL-NH_2-terminal FLAG construct. Perhaps the FLAG is not unharmful to some FasL epitopes.

Fielder *et al.* used untransfected cells as specificity controls for their FACS analysis. This procedure may lead to an underestimation of specific signals, as our data show that all the antibodies tested are crossreactive between human and murine FasL. Because many cell lines express low levels of FasL constitutively, or under certain culture conditions, specificity controls should be performed with isotype-matched antibodies and mock-transfected cells, as opposed to untransfected cells.

We also analyzed protein immunoblots analysis of 293T cells transiently transfected with human FasL, COS-7 cells transiently transfected with human FasL, and

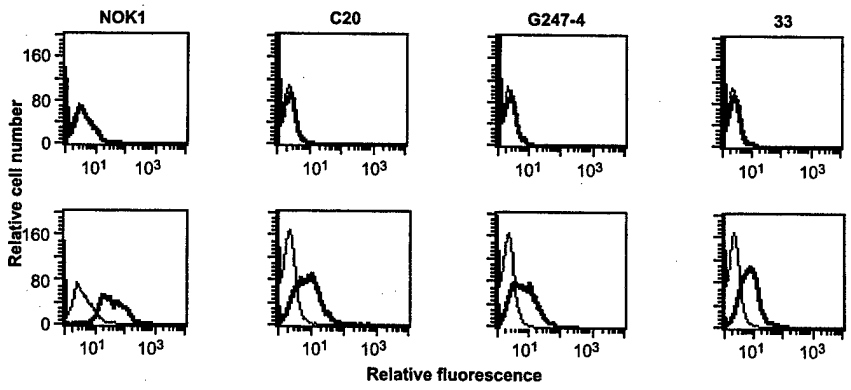

Fig. 3. FACS analysis of NIH 3T3 stably transfected with murine FasL, with the use of NOK-1, C20, G247-4, and 33 antibodies. Antibodies appear to cross-react between human and mouse FasL.

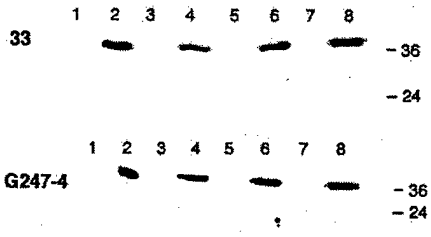

Fig. 4. Protein immunoblot analysis of FasL transfectants, with the use of both 33 and G247-4 antibodies. Lane 1: 293T cells, pcDNA3 empty-transfected. Lane 2: 293T cells, pcDNA3 hFasL-transfected. Lane 3: COS-7 cells pcDNA3empty-transfected. Lane 4: COS-7 cells pcDNA3hFasL-transfected. Lane 5: NIH-3T3 pSRa-72(N.1)empty-transfected. Lane 6: NIH-3T3 pSRa-72(N.1)mFas-L transfected. Lane 7: untreated Jurkat J77 cells. Lane 8: 8 hours PMA-activated Jurkat J77 cells.

NIH 3T3 stably transfected with murine FasL. To reveal FasL, we used mAb 33 and mAb G247-4. Both mAbs detected an ~37 kD band only in transfected cells (Fig. 4) and in PMA-treated, but not in PMA-untreated, Jurkat cells (8).

Fielder *et al.* find a uniform strong band reactive with the mAb 33 by protein immunoblot analysis in untransfected murine 293T cells, as well as in other human cell lines. As we detect mAb 33-reactive signals in transfected cells or in Jurkat only after PMA exposure, their finding is intriguing, and we have no explanation for it.

In conclusion, we find evidence that mAb 33 does recognize FasL in three different cell types, transiently or stably transfected with human or murine FasL, both by FACS and protein immunoblot analysis. Nevertheless, because of possible differences in specificity among the various available antibodies, we recommend the simultaneous use of several anti-FasL reagents. Constitutive FasL expression on in vivo and ex vivo normal thyrocytes has now been described by five different antibodies against FasL.

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3. Thyroid fragments (0.5 cm) were snap-frozen in isopentane, chilled at −150°C, and kept at −80°C until used. Serial cryostat thyroid sections (4 μm) were allowed to equilibrate to room temperature and exposed to acetone for 10 min before starting the peroxidase anti-peroxidase staining. Bound mAbs anti-FasL (NOK-2, PharMingen) and isotype matched control IgG, were detected by the labeled streptavidin-biotin staining technique (Vecstain quick universal Kit, Vector, Labs, Burlingame, CA), with the use of a biotinylated antibody to mouse immunoglobulins prediluted in TBS at room temperature for 20 min, followed by incubation for 10 min at room temperature with an peroxidase-conjugated steptavidin. Primary antibodies were added, without washing, to the tissue preparations after incubation for 20 min with antibody to human serum. Binding was revealed by 3,3′-diaminobenzidine tetrahydrochloride (DAB) colorimetric substrate for 2 min. Haematoxylin aqueous formula was used as a counterstain.
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5. M. Tanaka, T. Suda, T. Takahashi, *EMBO J.* **14**, 1129 (1995).
6. COS-7 and 293T cells were transiently transfected respectively by the DEAE-dextran and the Calcium-phospate methods with a pcDNA3FasL expression vector coding for a full-length human FasL or with a pcDNA3 empty vector. Transfection cells were stained 48 hours later with anti-FasL antibodies NOK-1 (PharMingen, Lot. M021271) (5 μg/ml), C20 (Santa Cruz Biotechnology, Lot. H145) (1 μg/ml), G247-4 (PharMingen, Lot M021163) (5 μg/ml), clone 33 (Transduction Laboratories, Lot 2) (5 μg/ml), or an isotype-matched control antibody, followed by a FITC-coupled sheep antibody to mouse or a FITC-coupled donkey antibody to rabbit Ig–conjugated antibody (Amersham). Cells were analyzed with a FACScan (Becton Dickinson).
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8. We also used aliquots of the transfected cell lines in protein immunoblot experiments. Briefly, cells were lysed (lysis buffer: 50 mM tris, pH 7.6, 150 mM NaCl, 0.5% NP-40, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF) and an equal amount of proteins (50 to 100 μg) for each sample was loaded on a 12% SDS-PAGE, blotted against nitrocellulose filter, and analyzed with the CD95L antibodies clone 33 (0.3 μg/ml) or clone G247-4 (2 μg/ml) with the use of HRPO-coupled sheep antibody to mouse Ig and enhanced chemoluminescence (Amersham).

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Figure 3

3: FasL expression, detected by clone N20, does not increase under conditions of apoptosis in Myc83 cells.

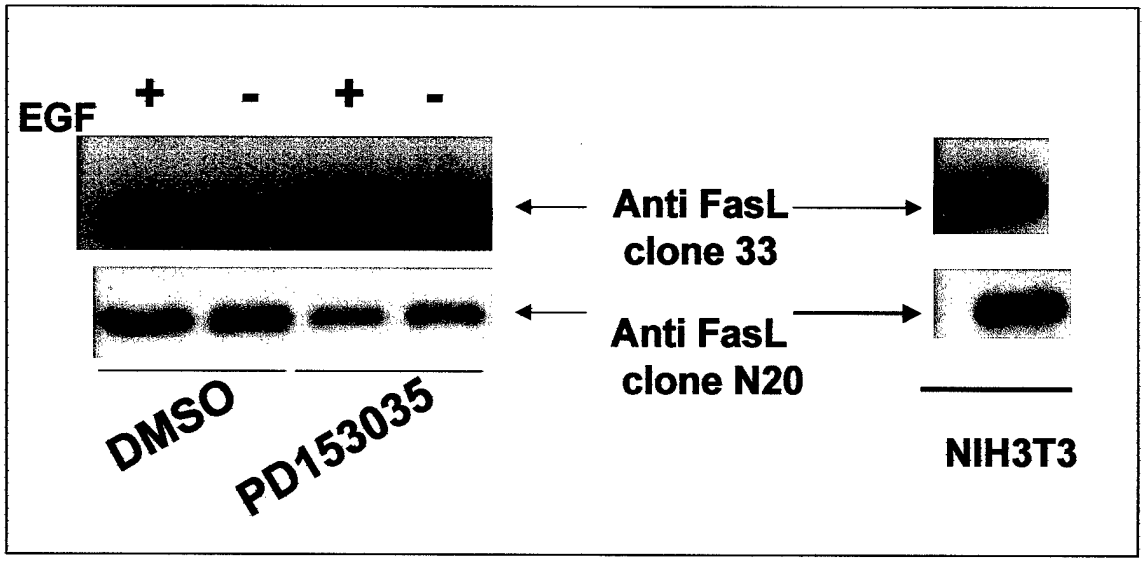


Figure 4

4:Presence of a constitutively activated Akt (Myr-Akt) prevents the PD153035-induced increase of FasL expression in Myc83.

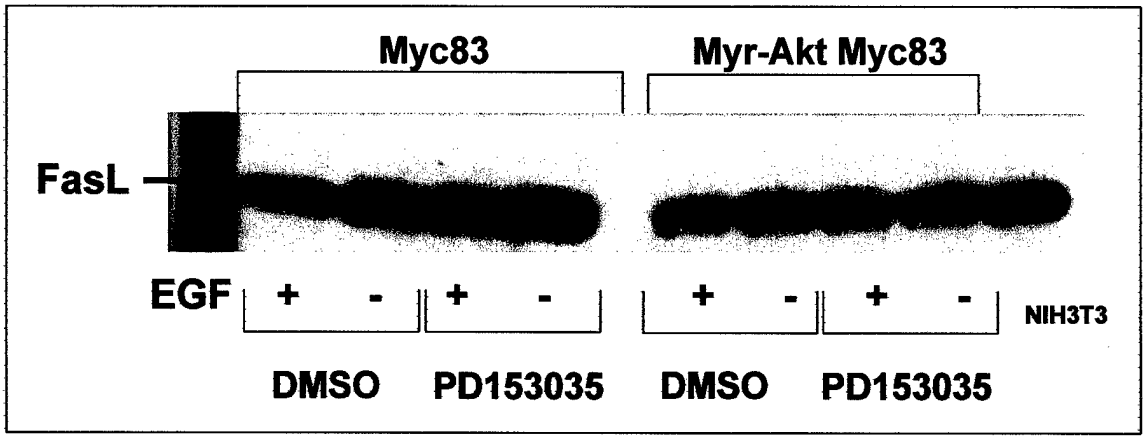


Figure 5a

5a: Treatment with the Calcium Chelator BAPTA-AM prevents the activation of Akt in Myc83 cells.

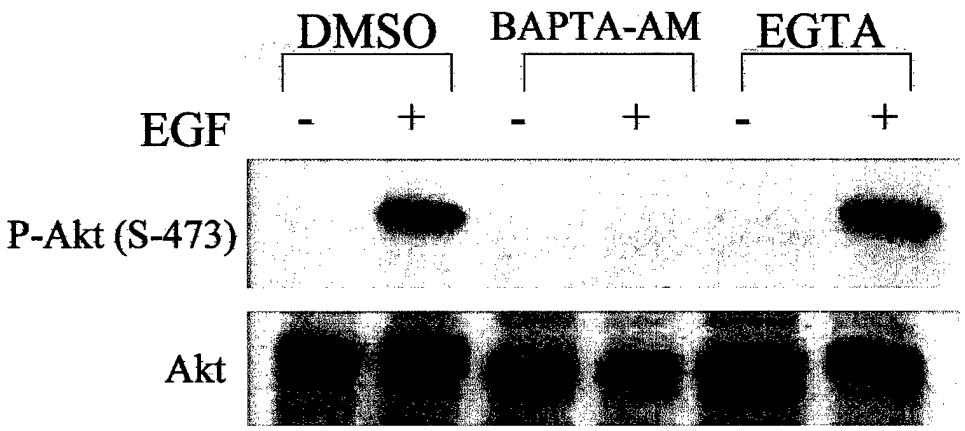


Figure 5b

5b: Treatment with the calmodulin inhibitor W-7 but not the control W-12 prevents the activation of Akt in Myc83 cells.

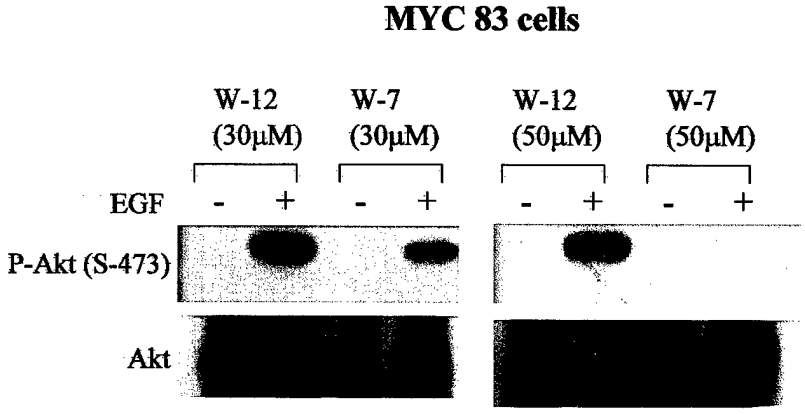
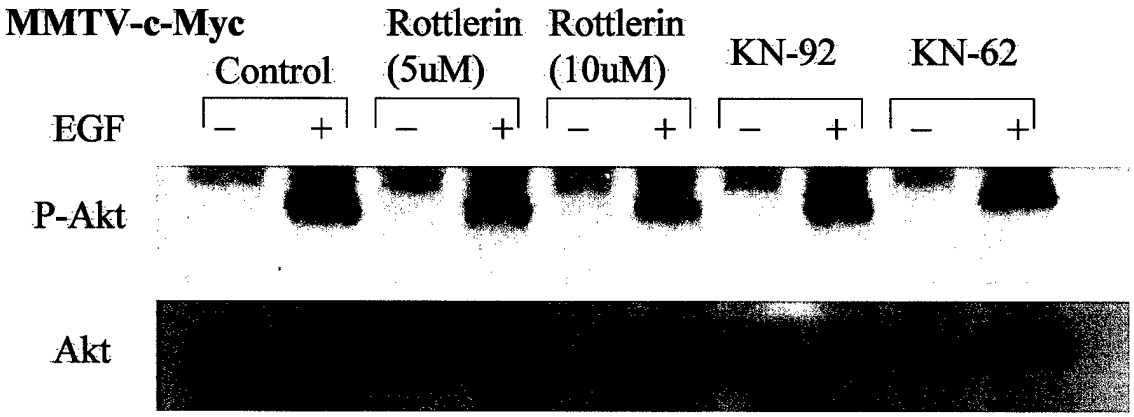


Figure 6

6: Treatment with the CaM kinase III inhibitor, rottlerin, but not other CaM kinase inhibitors, decreases the activation of Akt in Myc83 cells.



APPENDIX B
Papers, Abstracts, Accomplishments

PAPER 1: Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-XL upregulation. Danica Ramljak, Christine M. Coticchia, Tagvor G. Nishanian, Motoyasu Saji, Matthew D. Ringel, Suzanne D. Conzen, Robert B. Dickson. *Experimental Cell Research*, Submitted July 2002

ABSTRACT 1: Epidermal growth factor receptor signaling inhibits c-Myc-induced Apoptosis through Activation of Akt, Erk and upregulation of Bcl-xL in mouse mammary carcinoma cells. Danica Ramljak, Christine M. Coticchia and Robert B. Dickson. Keystone Conference on Molecular Mechanisms of Apoptosis . Keystone, Colorado. January 2001

ABSTRACT 2: The role of c-Myc overexpression in sensitization of mammary epithelial cells to apoptosis. Christine M. Coticchia and Robert B. Dickson. Era of Hope Conference, Orlando, Florida. September 2002.

ACCOMPLISHMENT 1: Completion of comprehensive Exams: August 2001

Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-x_L upregulation in mammary epithelial cells

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Running title: Survival pathways in mammary epithelial cells

Supported by NIH grants 1RO1CA72460 and 1R01AG496 to Robert B. Dickson.

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Keywords: Akt; Erk; Bcl-x_L; EGFR; c-Myc

Summary

Currently, limited information exists linking epidermal growth factor receptor (EGFR) signaling to cellular survival in mouse and human mammary carcinoma cells. In earlier studies, others and we have established that activation of EGFR can promote survival in association with upregulation of Bcl-x_L. However, the mechanism responsible for upregulation of Bcl-x_L is unknown. For the current studies we have chosen pro-apoptotic, c-Myc-overexpressing murine mammary epithelial cells (MMECs) derived from MMTV-c-Myc transgenic mouse tumors. We now demonstrate that EGFR activation promotes survival through Akt and Erk1/2. Blockade of EGFR kinase activity and the PI3-K/Akt and MEK/Erk pathways with pharmacological inhibitors resulted in a significant induction of cellular apoptosis, paralleled by a down-regulation of both Akt and Erk1/2 proteins. Consistent with a survival-promoting role of Akt, we observed that constitutively activated Akt (Myr-Akt) inhibited apoptosis of pro-apoptotic, c-Myc-overexpressing cells following the inhibition of EGFR tyrosine kinase activity. In addressing possible downstream effectors of EGFR through activated Akt, we detected significant upregulation of Bcl-x_L protein, suggesting this pro-survival protein is a potentially novel target of Akt in MMECs. The responsible mechanism is being explored. Our findings are of importance because of the recently emerging role of Bcl-x_L as a potential prognostic marker in breast cancer.

Introduction

The c-*myc* gene is thought to play an important role in the onset and progression of breast cancer, where it is commonly amplified and/or overexpressed (1, 2). Depending upon the availability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through poorly defined mechanisms. Previously, we demonstrated that an EGFR-mediated survival signaling pathway(s) inhibited apoptosis in c-Myc-induced transgenic mouse mammary tumors (3). Comparison of tumors and cell lines derived from bitransgenic *tgf α/c-myc* mice to those from single transgenic *c-myc* mice indicated that only the latter model contained a significant fraction of apoptotic cells (3), suggesting that TGFα protects c-Myc-overexpressing cells from apoptosis *in vivo*. Further *in vitro* studies of c-Myc-overexpressing mammary tumor-derived cells confirmed that EGFR ligands, acting through EGFR tyrosine kinase activity, suppressed apoptosis and upregulated the survival molecule Bcl-x_L, at both mRNA and protein levels (4). However, it was not clear which pathways downstream of EGFR are responsible for these effects.

Presently, there is limited information on the signaling pathways linking EGFR to the regulation of cellular survival in mouse and human mammary epithelial and carcinoma cells. However, studies in some non-mammary epithelial cells (hepatic carcinoma cells and keratinocytes) have identified two survival pathways downstream of EGFR: phosphatidylinositol 3-kinase (PI3-K)/Akt and extracellular signal regulated kinase (Erk1/2). In most cases, the PI3-K/Akt pathway delivers the most potent survival signal downstream of EGFR (5, 6).

Akt is a serine-threonine kinase, downstream of PI3-K, which delivers strong survival signals in many cell types (7-9). Both growth factors and integrins activate Akt through activation of PDK1 and putative PDK2 kinase that subsequently phosphorylate Akt at Thr308

and Ser473 respectively (10). There are several isoforms of Akt (Akt1, Akt2 and Akt3); each has been shown to be expressed at different levels in various tissues (11). The targets of Akt in epithelial cells, including mouse and human breast cells, include proteins involved in cell growth, metabolism and apoptosis. The Akt targets involved in apoptosis include Bad, a pro-apoptotic member of the Bcl-2 family of proteins (12), caspase 9 (13), and the forkhead transcription factor (14). Recently, Akt also has been reported to upregulate the expression of anti-apoptotic proteins in lymphoid cells such as Bcl-x_L (15), Bcl-2 (16) and Mcl-1 (17). Akt activates NF-κB in both fibroblasts and epithelial cells (18, 19).

In most cell types, both growth factors and integrins are capable of activating the MAPK/Erk pathway. Of particular relevance to our studies, the MAPK/Erk pathway was previously shown to convey survival signals in response to EGF (20, 21). Recently, it has been shown that the PI3-K/Akt and the MAPK/Erk pathways can cooperate in the inhibition of Bad in some cell types (22). However, the pro-survival targets and the interactions of EGFR-activated PI3K/Akt and MAPK/Erk pathways have not been established in MMECs, human breast epithelial cells or human carcinomas. Our preliminary data indicated that Bad is well expressed in MMECs; however, the phosphorylation status of endogenous Bad was difficult to determine due to the lack of reliable antibodies. With the emerging role of activated EGFR in breast cancer, we believed it would be important to determine the anti-apoptotic targets of EGFR-stimulated Akt and Erk, in MMECs and human breast cancer cells.

In our investigations to determine which survival molecules downstream of the EGFR are responsible for upregulation of Bcl-x_L and inhibition of c-Myc-mediated apoptosis in MMECs we show that constitutively activated Akt and Erk provide protection from c-Myc-mediated apoptosis in association with upregulation of the Bcl-x_L protein. These novel findings indicate

that Akt, possibly together with Erk, could affect the expression levels of Bcl-x_L and lead to apoptosis inhibition in MMECs. This is completely different from observation in fibroblasts in which Akt was not able to affect Bcl-x_L expression, indicating a major difference between epithelial cells and fibroblasts.

Experimental procedures

Cell Culture and Viral Infection - Myc83 cells (derived from an MMTV-*c-myc* transgenic mouse mammary tumor in our laboratory), Comma D (immortalized mouse mammary epithelial cells obtained from D. Medina, Baylor College of Medicine) (23) were maintained in a humidified 5% CO_2 environment in complete media containing: IMEM (Gibco-BRL, Gaithersburg, MD), 2.5% fetal calf serum (FCS), 10 ng/ml EGF (Upstate Biotechnology Incorporated, Lake Placid NY) and 5 μg/ml insulin (Biofluids, Rocville, MD). Myc83 cells were selected as a model because of their high propensity to apoptose after removal of EGF. Comma D cells (with normal c-Myc levels and mutated p53) were used to compare the potency of EGF in activating both Akt and Erk1/2 in MMECs with different c-Myc levels. Retroviruses for Myr-Akt (obtained from N. Hay, University of Illinois) (24) were made by transient transfection of retroviral pBabePuro-Myr-Akt using Effectene Transfection Reagent (Qiagen, Valencia, CA) into amphotrophic Phoenix cells (a gift of Dr. Gary Nolan, Stanford University, Palo Alto, CA). Viral supernatants were collected and filter purified. Myc83 cells were infected with pBabePuro vector only or pBabe Myr-Akt in the presence of 4 μg/ml of polybrene, and infected stable clones were selected with puromycine (Sigma, St. Louis, MO). Clones were pooled for further analysis. All retrovirally transduced cells were grown in complete media, with or without addition of EGF. When testing sensitivity to apoptosis, cells were grown in IMEM only, without EGF, FCS or insulin, or in IMEM with EGF only.

Antibodies and Reagents - Rabbit polyclonal anti-total Akt, anti-total Akt phospho-specific antibodies recognizing Ser473 and Thr308; anti-Erk1 and Erk2 and anti-Erk1/Erk2 phospho-specific antibodies, Bad and phospho-Bad (Ser 112 and Ser 136) were from Cell Signaling Biolabs (Beverly, MA). Anti-Akt1, anti-Akt2 and Akt3 were from Upstate Biotechnology (UBI), mouse monoclonal

anti-α-tubulin and anti-Bcl-2 were from Neomarkers, Fremont, CA); anti-Bcl-x_L (H-62), anti-14-3-3. (C-16), rabbit polyclonal poly(ADP-ribose) polymerase (PARP) (H-250), anti-GSK-3β(0011-A), anti-p85 PI3-K (Z-8) and anti-Raf-1(C-12) were from Santa Cruz (Santa Cruz, CA). The ECL detection reagent was from Amersham, (Arlington Heights, IL); PI3-K inhibitor LY294002, and EGFR inhibitor PD153035 were from Biomol Research Laboratories (Plymouth Meeting, PA); and MAPK kinase (MEK) inhibitor U0126 was from Cell Signaling (Beverly, MA).

EGF Stimulation and Treatments with Inhibitors – Myc83 and Comma D cells (1-2 x 10^6/ml) were grown in 10 cm^2 Petri dishes in complete IMEM media containing 2.5% FCS, 10 ng/ml EGF (Upstate Biotechnology Incorporated, Lake Placid NY) and 5 μg/ml insulin (Biofluids, Rocville, MD) until cells reached 60-70% confluence. Complete media was removed, cells were washed with 1X PBS to remove serum and growth factors, and synchronized by serum starvation (grown in IMEM media with 0.1% fetal bovine serum) for 24 hours. After two washes with 1X PBS, the cells were stimulated with IMEM media containing 10 ng/ml of EGF for 1, 2.5, 5, 10 and 20 minutes, washed twice with 1X PBS and lysed in buffer (Cell Signaling Biolabs) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1mM β-Glycerophosphate, 1mM Na_3VO_4, 1 μg/ml leupeptin and 1mM phenylmethylsulfonyl fluoride (PMSF) for protein analysis. Insoluble material was removed by centrifugation. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) and samples were stored at -80^0C until used for immunoblotting and *in vitro* kinase assays.

In order to test the requirement of active EGFR for EGF-induced Akt and Erk1/Erk2 activation, the synchronized Myc83 cells (as described above), were pretreated with 1 μM of PD153035 EGFR tyrosine kinase inhibitor for three hours, followed by a treatment with 10 ng/ml of EGF at 2.5, 5 and 10 minutes, as described above. The control Myc83 cells (-PD153035) were pretreated with an equal volume (10 μL) of DMSO and later stimulated with 10 ng/ml of EGF for 2.5 and 5 minutes. Comma D cells were pretreated with the same concentration of PD153035 (1 μM) and for the same time (3 hours) as Myc83 cells, and they were similarly stimulated with 10 ng/ml of EGF for 1, 2.5 and 5 minutes. Control Comma D cells (-PD153035) were treated with EGF for 1, 2.5, 5, 10 and 20 minutes. Proteins were analyzed for the activity of both Akt and Erk1/Erk2 by immunoblotting, as described in the Western blotting portion of the Experimental Procedures.

Induction of apoptosis by prolonged treatment with inhibitors of PI3-K, MEK and EGFR - Myc83pBabePuro and Myc83-Myr-Akt cells were grown in complete IMEM media to 70-80% confluence in 10 cm^2 plates, and then the complete media was replaced with IMEM media containing the following: 10 ng/ml EGF (without serum and insulin); 50 μM LY294002 (Biomol) with and without the EGF; 10 μM U0126 (Cell Signaling Biolabs) with and without EGF, 1 μM PD153035 (Biomol), with and without the EGF. Cells were usually grown for 48 hours, but in some cases cells were harvested after only 24 hours. In one set of plates, cells were lysed for protein analysis, as described below, while another set of cells, treated equally, was used for evaluation of apoptosis by Hoechst staining.

Measurement of apoptosis- Apoptosis was evaluated by immunoblotting of PARP protein cleavage, as described in the *Western blotting* part of the Experimental Procedures, and by

Hoechst staining. Briefly, Hoechst staining was performed as follows: after 48 hours of treatment with LY294002, U1026 and PD153035, all adherent and floating cells were collected. Samples were centrifuged for 8 minutes at 1000 g at 4^0C. Supernatants were discarded and cell pellets were resuspended in 1X PBS containing formaldehyde and NP-40, and stained with 10 μg/ml of Hoechst 33258 dye for apoptotic analysis. At least 500 cells per treatment group were counted with a hemocytometer and evaluated for the presence of condensed nuclei and overall apoptotic appearance.

Akt Kinase Assay- Protein lysates from control and EGF-stimulated Myc83 and Comma D cells (0, 1, 2.5, 5, 10 and 20 minutes) were lysed in the buffer described above. Akt kinase activity was analyzed using an Akt Kinase Assay Kit (NEB), which employs GSK-3 as a substrate, according to the manufacturer's instructions. In short, 300 μg of cellular protein was immunoprecipitated with a total Akt antibody immobilized to agarose beads (NEB) at 4^0C overnight. Immunoprecipitated Akt protein on beads was washed twice with 1 ml of lysis buffer (described above) and once with kinase buffer (25 mM Tris, pH 7.5; 5-mM β-Glycerolphosphate, 2 mM DTT, 0.1 mM Na_3VO_4 and 10 $MgCl_2$). The beads were then resuspended in 40 :l of kinase buffer containing the Akt protein substrate (1μg of GSK-3 α/β fusion protein), supplemented with 200 μM of ATP. The assay was carried out according to the manufacturer's instructions (NEB), and protein samples were loaded on 12% SDS-PAGE gel (Novex) and Western immunoblotting was performed, as described below. The membranes were probed with a phospho-specific antibody recognizing GSK-3α-P (at 1:1000 dilution) when phosphorylated by Akt on serine 21 (NEB). The secondary antibody utilized at 1:2000 was a rabbit polyclonal (NEB). Blots were developed using ECL reagents (Amersham) and exposed to ECL film

(Amersham). The intensity of the bands was quantified using a Chemilmager 5500 (Alpha Innotech Corp., San Leandro, CA).

Western blotting- Cell lysates containing a total of 10-15 μg of protein were used for the Western blot analysis. A PhosphoPlus^{R} MAPK antibody kit was used according to the manufacturer's recommendations (Cell Signaling), to determine Erk activation by immunoblotting in all experimental and control cells. Cellular extracts were analyzed with antibodies against total Erk1/2 (p44 and p42) and with phospho-specific antibodies against phospho-p44/42 (Thr202/Tyr204) (Cell Signaling). In addition to Akt kinase assays (see above) the total activity of Akt was determined using phospho-specific antibodies recognizing Akt specifically phosphorylated at Ser^{473} or Thr^{308} (Cell Signaling). Akt protein expression was determined by using an antibody recognizing total Akt independent of phosphorylation status (Cell Signaling).

All samples for Akt, Erk1/2, p85 PI3-K, Raf-1 and PARP were analyzed on 8% SDS-page gels (Invitrogen, Carlsbad, CA). Those probed for GSK-3β utilized 10% SDS-PAGE gels, while samples analyzed for Bcl-2, 14-3-3. and Bcl-x_L were tested on 12% SDS-PAGE gels (Novex). Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) by electroblotting. After transfer, membranes were stained by Ponceau protein stain (Sigma) to test for equal loading. The membranes were then washed with 1X phosphate buffer saline /0.1% Tween 20 (PBST) 3 X 10 minutes. After washing, membranes were blocked with 2% bovine serum albumin (BSA) in for 1 hour. After incubation in primary antibody overnight at 4^0C, all blots were washed for 3 X 10 minutes with PBST and probed for 1 hour with the corresponding secondary antibody (anti-mouse or anti-rabbit labeled with horseradish peroxidase; from

Amersham, Arlington Heights, IL or from NEB). After three repeated washes in PBST the blots were developed using a chemiluminescence ECL kit (Amersham) and exposed to X-ray film (Amersham). On several occasions, in order to test equality of loading, membranes were stripped and reprobed with an antibody recognizing α-tubulin, a 57 kD protein (Neomarkers).

Results

EGF is potent activator of Akt in mouse mammary epithelial cells - Our previous studies showed that activation of the EGFR by either EGF or TGFα delivers a potent survival signal to mouse mammary epithelial cells overexpressing c-Myc, both *in vivo* and *in vitro* (3). However, it was not clear which survival pathways downstream of the EGFR were responsible for the inhibition of apoptosis. In the present work, we show that in the absence of serum, EGF stimulation of both Myc83 (MMECs cells derived from mouse transgenic for c-Myc) and Comma D (immortalized MMECs) significantly activate Akt, a potent survival molecule in fibroblasts and several other epithelial cell types (8). Akt is activated by 10 ng/ml of EGF, within one minute, in both Myc83 (Figure 1a) and Comma D cells (Figure 1c). Maximum Akt activation in Myc 83 cells was seen at 5 minutes, followed by a decrease to basal activity by 10 minutes (Figure 1a). Similar results were obtained for Comma D cells (Figure 1d). EGF activation of Akt resulted in the phosphorylation of Ser473 (Figure 1a and 1d) and Thr308 (data not shown). Phosphorylation of both sites is required for full activation of Akt (9). As expected, the EGF treatment did not affect the expression levels of total Akt protein in either cell line as shown by immunoblotting using anti-Akt antibody recognizing all three Akt isoforms (Figure 1b and 1e). The activity of Akt following EGF stimulation was confirmed by an Akt kinase assay, using GSK-3α fusion protein as a substrate (Figure 1c and 1f). Akt has been shown to phosphorylate GSK-3α at serine 21 (25). By using an antibody that specifically recognizes phospho-GSK-3α at serine 21, we confirmed the phosphorylation of GSK-3, a known target of activated Akt, in both Myc83 (Figure 1c) and Comma D (Figure 1f).

In order to determine which of the three major Akt isoforms (Akt1, Akt3 and Akt3) are expressed and activated after EGF treatment in MMECs, we evaluated their expression levels in Myc83 and Comma D by using antibodies specifically recognizing each isoform (UBI). We detected significant expression of Akt1 and Akt2 in all MMECs analyzed (data not shown), while expression of Akt3 could not be detected in any of the cells by the antibody used in this study (data not shown).

Activation of Erk1/Erk2 by EGF parallels Akt activation - Although in the majority of cells the PI3-K/Akt pathway has been shown to deliver a stronger survival signal compared to the MEK/Erk pathway, in some epithelial cells, both pathways appear to be equally important in delivering survival signals (26). Therefore, we next determined the status of Erk1/Erk2. The results show that EGF is capable of specifically activating Erk1/Erk2 in both cell lines (Figure 2a and 2c), within a similar time frame, as shown for Akt (Figure 1a and 1d). In each cell line, the highest Erk1/Erk2 activity was detected at 2.5 and 5 minutes; similar to Akt, the activity declined by 10 minutes. In Myc83 cells, the signal for Erk1 was the stronger of the two isoforms (Figure 2a), while in Comma D cells, Erk2 was much more activated (Figure 2c). The expression of total protein levels for Erk1 and Erk2 (Figure 2b and 2d) was unchanged by EGF treatment of either cell line.

Inhibition of EGFR tyrosine kinase prevents activation of both Akt and Erk1/Erk2 in c-Myc-overexpressing MMECs - In order to test the requirement of activated EGFR kinase in the activation of Akt, Erk1 and Erk2, we blocked EGFR tyrosine kinase with PD153035, followed by EGF treatment. In both Myc83 and Comma D cells, inhibition of EGFR kinase activity

completely abolished EGFR-mediated activation of Akt (Figure 3a and 3e), without affecting total protein levels (Figure 3b and 3f). As expected, EGF treatment strongly activated Akt in cells treated with vehicle (DMSO) (Figure 3a and 3e). However, only in Myc83 cells was blockade of the EGFR tyrosine kinase activity capable of inhibiting activation of Erk1/Erk2 (Figure 3c), without affecting the expression levels of Erk1/Erk2 proteins (Figure 3d). Interestingly, inhibiting EGFR with PD153035 in Comma D cells did not prevent activation of Erk1/Erk2 by EGF (Figure 3g). Regardless of the presence or absence of PD153035, both Erk1 and Erk2 were strongly activated within 1 minute, with increasing activity up to 5 minutes; however, the expression of neither Erk1 nor Erk 2 was affected (Figure 3h). The mechanism(s) responsible for this effect is being investigated.

Prolonged inhibition of PI3-K activity leads to apoptosis and a decrease in protein levels of both Akt and Erk1/Erk2- Recently, some evidence in the literature suggests that the Akt protein is cleaved by caspases late in the apoptotic process induced by UV irradiation, Fas ligation, and etoposide in human Jurkat cells (27). Therefore we tested the status of the Akt protein in apoptotic Myc83 cells, following the prolonged inhibition (48 hours) of PI3-K activity with 50 μM of LY294002. In these experiments, we compared levels of Akt protein in LY294002-treated and untreated Myc83 cells grown in the presence or absence of EGF. First, by evaluating cleavage of the full length form PARP (an early indicator of apoptosis) we found that prolonged treatment with LY294002 leads to apoptosis of Myc83 cells, regardless of the presence or absence of EGF (Figure 4a). In the same protein lysates, apoptosis was paralleled by a prominent decrease in Akt levels, independent of the presence of EGF in the growth media (Figure 4b). The decrease in Akt phosphorylation was detected in the sample treated with LY294002 in the

absence of EGF (Figure 4c). Furthermore, in the same samples where decrease in the Akt protein expression and phosphorylation was detected, expression of α-tubulin protein (loading control) was unchanged (Figure 4d), suggesting that the prolonged inhibition of the PI3-K in c-Myc-overexpressing cells specifically caused downregulation and proteolysis of the Akt protein.

In order to evaluate how early in the apoptotic process this downregulation of Akt was occurring, we analyzed protein lysates of Myc83 cells treated with the same concentration of LY294002 (50 μM), with and without EGF, for periods of 24 and 48 hrs. Again, PARP cleavage immunoblotting was used to evaluate apoptosis (Figure 5a), and the same protein samples were further evaluated for the expression of Akt. Interestingly, as early as 24 hrs after treatment with LY294002, the expression of Akt protein decreased, (Figure 5b) preceding the onset of apoptosis, detected by PARP cleavage (Figure 5a). However, by 48 hrs, when a large percentage of the cells underwent apoptosis, as determined by PARP cleavage (Figure 5a), the decrease in Akt protein level was more prominent (Figure 5b). The mechanism responsible for decreased steady state levels of Akt is currently under investigation.

To determine if the prolonged inhibition of PI3-K over 24 and 48 hours specifically is causing Akt downregulation, we evaluated the status of Erk1 and Erk2, Raf-1 (previously shown, together with Akt, to be cleaved late in the apoptotic process in Jurkat cells) (27), and p85 PI3-K. Similar to Akt, the expression of both Erk1 and 2 proteins decreased in the same protein lysates in which we detected the downregulation of the Akt, following treatment with LY294002 (Figure 5c). The decrease in Erk1 was more pronounced, in comparison to Erk2 (Figure 5c). In contrast to the reported decrease in expression of Raf-1 protein in apoptotic Jurkat cells (27), we did not

detect any change in expression of Raf-1 (Figure 5d) in apoptotic Myc83 cells. However, expression of p85 PI3-K was slightly decreased, 48 hrs after EGF removal, and in LY294002-treated Myc83 cells, in the absence of the EGF for 48 hrs (Figure 5e). The absence of Raf-1 protein downregulation further implicates the involvement of PI3-K/Akt pathway.

Downregulation of Akt protein is specific only for apoptosis induced through prolonged inhibition of PI3-K activity - In order to determine if the downregulation of Akt protein is simply the result of ongoing apoptosis in Myc83 cells, regardless of which of the survival pathways has been blocked, we treated Myc83 cells with 1 μM of PD153035, a specific inhibitor of the EGFR; with 10 μM of U0126 (a MEK inhibitor), and with 50 μM of LY294002 (PI3-K/Akt inhibitor) for 48 hrs. The samples were then evaluated for apoptosis by Hoechst staining and by PARP cleavage, as well as by immunoblotting for Akt protein expression. In the presence of EGF, only 2-3% of Myc83 cells underwent apoptosis, however, when EGF was removed, the number of apoptotic cells increased to 9% (Figure 6a). When cells were treated with PD153035, in the presence of EGF, a total of 42 % of the cells underwent apoptosis (Figure 6a), while after removal of the EGF, 49% of the cells were apoptotic. In the presence of EGF and U0126, a total of 15% of the cells underwent apoptosis (Figure 6a), while after removal of EGF almost 23% of the cells were apoptotic (Figure 6a). A total of 22% of the Myc83 cells treated with EGF and LY294002 underwent apoptosis after 48 hrs; following EGF removal, treatment with LY294002 caused 33% of the cells to apoptose (Figure 6a). In addition, we treated a separate set of Myc83 cells with the same inhibitors, under the same conditions for 48 hrs, and then lysed the cells for PARP protein analysis by immunoblotting. As expected, the results of PARP cleavage (Figure 6b) confirmed the apoptosis results with Hoechst staining (Figure 6a).

Interestingly, only those cells treated with an inhibitor of PI3-K (LY294002) for 48 hours exhibited a significant decrease in the expression of the Akt (Figure 6c). However, regardless of the apoptosis occurring in cells treated with other pharmacological inhibitors (PD153035 and U0126), the expression of Akt protein was not affected (Figure 6c).

Constitutively activated Akt protects MMECs overexpressing c-Myc from apoptosis- In order to further confirm the potential importance of Akt to deliver a survival signal downstream of EGFR, we retrovirally transduced constitutively activated Akt (Myr-Akt) into the Myc83 cells. In contrast to the parental Myc83 cells, previously shown to undergo apoptosis within 48 hrs after the removal of EGF and serum, Myc83-Myr-Akt cells were highly resistant to apoptosis upon the removal of EGF and serum from the growth media, for up to five days (data not shown). The apoptotic response was evaluated by measuring PARP cleavage (Figure 7a). PARP cleavage was detected in apoptotic, pBabe-Myc83 cells treated with PD153035 for 48 hrs, while in Myc83-Myr-Akt cells, treated under the same conditions for 48 hrs, cells were protected from c-Myc-mediated apoptosis regardless of the presence of EGF or PD153035 (Figure 7b).

Furthermore, the apoptotic profile of Myc83-Myr-Akt cells was additionally evaluated by Hoechst staining following treatment with 1 μM of PD153035, 10 μM of U0126 and 50 μM of the LY294001 for 48 hrs. Again, constitutively active Akt in Myc83-Myr-Akt cells almost completely protected the cells from apoptosis resulting from the inhibition of EGFR kinase activity by PD153035 (Figure 7c). After treatment with PD153035, in the presence of EGF, only 8% of the Myc83-Myr-Akt cells underwent apoptosis, in comparison to 42.4% of Myc83 control cells (Figure 7c). After the removal of EGF, in the presence of PD153035, a similar percentage of Myc83-Myr-Akt cells

underwent apoptosis (7.4%), compared to 48.4% of control Myc83 cells in the same experiments. These data suggest that, despite the prolonged inhibition of EGFR kinase activity, the presence of constitutively activated Akt provides full protection from apoptosis in c-Myc-overexpressing MMECs.

Interestingly, constitutively activated Akt protected from apoptosis only 27% of the Myc83-Myr-Akt cells, in comparison to 22% of Myc83 in the presence of EGF plus the inhibitor of the PI3-K pathway (LY294002). Under the same conditions (in the presence of the LY294002), when EGF was removed, only 29% of Myc83-Myr-Akt cells underwent apoptosis, compared to 32.5% of Myc83 cells. This result is not surprising, due to the fact that the myristolyation signal has been shown to decrease following prolonged inhibition of the PI3-K activity in fibroblasts, leading to a decrease in Akt activity (24). Also, because we detected proteolysis of endogenous Akt protein under the same condition of prolonged inhibition of PI3-K activity for 48 h (Figure 7c), Myr-Akt might be targeted to proteolysis as well. As expected, the constitutively activated Akt in Myc83-Myr-Akt did not provide protection from apoptosis induced from prolonged inhibition of the MEK/Erk pathway with U0126 (Figure 7c).

The mechanism by which constitutively activated Akt inhibits apoptosis *in c-Myc-overexpressing cells involves upregulation of Bcl-x_L* - In fibroblasts and haematopietic cells, activated Akt has been shown to inhibit apoptosis, mostly through mechanisms involving the inhibition of the pro-apoptotic Bad protein (12, 8), caspase 9 (13), and inhibition of release of cytochrome c (24). However, little information is available on the mechanism whereby Akt inhibits apoptosis in

MMECs and human breast cells, and the mechanism by which Akt inhibits c-Myc-mediated apoptosis in general.

In our previous work we observed that one of the molecules upregulated by activation of EGFR in Myc83 cells is the pro-survival Bcl-x_L protein, both at the mRNA and protein levels (4). Therefore, in order to explore if the mechanism whereby activated EGFR inhibits c-Myc-induced apoptosis involves Akt in our model, we analyzed the status of Bcl-x_L in parental Myc83 cells and Myc83-Myr-Akt cells grown in the presence of complete media. Western blot analysis detected a significant, 2-fold increase in Bcl-x_L protein levels in Myc83-Myr-Akt cells, in comparison to Myc-83 cells transfected with vector only (Figure 8a). Currently, we are further investigating the possible mechanism by which Akt and possibly Erk may upregulate Bcl-x_L.

In addition, we analyzed the expression of the pro-survival Bcl-2 (Figure 8b), and 14-3-3 (data not shown), other survival molecules, and no changes in expression were detected. Furthermore, we evaluated expression of the pro-apoptotic Bad protein, a known target of Akt. Although Bad expression levels did not differ between Myc83 and Myc-Myr-Akt cells (data not shown), it was difficult to determine the phosphorylation status of endogenous Bad in these cells by commercially available antibodies.

Discussion

Increased activation of EGFR (28-31) and dysregulated expression of c-Myc (1, 2) are both commonly observed in human breast cancers. We previously described the dramatic interaction of these two tumor-associated aberrations in a bitransgenic model of human mammary cancer. Our studies showed that EGFR strongly suppressed c-Myc-mediated apoptosis by pro-survival signaling (3). Signaling pathways linking EGFR to cellular survival in the context of inhibition of the pro-apoptotic state induced by c-Myc are not well defined in either mouse mammary or human breast cancer epithelial cells. We now provide evidence that EGF activates Akt/PKB and Erk1/2 in MMECs that overexpress c-Myc, and that both pathways inhibit c-Myc-mediated apoptosis. These results may be specific for epithelial cells because in fibroblasts, EGF did not cause significant activation of Akt, nor was it able to protect cells from apoptosis (8).

Akt has been shown previously to inhibit apoptosis in fibroblasts, neuronal cells, hematopoietic, and some epithelial cells (8, 9), but its role in apoptotic inhibition of mouse mammary and human breast cancer cells overexpressing c-Myc is unexplored. Recently, EGF has been reported to activate Akt in rat fetal hepatocytes (33), in two human breast cancer cell lines, MCF-7 and T47D (33). Our results are among the first demonstrating the importance of Akt activation in conveying survival signals downstream of EGFR in MMECs overexpressing c-Myc.

In the current study we show that constitutively activated Akt inhibits apoptosis, while upregulating protein levels of the pro-survival molecule Bcl-x_L. This finding is consistent with our previous data, where we showed that EGF delivers survival signaling in these same c-Myc-overexpressing MMECs in association with upregulation of Bcl-x_L, both at the mRNA and protein levels (4). Similarly, in mouse hepatocytes EGF exerts its anti- apoptotic action partially

through upregulation of Bcl-x_L (34), and EGF receptor signaling inhibits keratinocyte apoptosis through increased expression of endogenous mRNA and protein levels of Bcl-x_L (35). However, it is not clear, which signaling molecules downstream of EGFR are involved in the upregulation of Bcl-x_L. Our results here are the first indication that Bcl-x_L could be upregulated by Akt in models of human breast cancer. Currently, we are in the process of further investigating this finding in attempt to elucidate the mechanism by which Akt upregulates Bcl-x_L.

Based on our work it seems that, contrary to what has been found in fibroblasts, Akt can regulate Bcl-x_L expression in mouse mammary epithelial cells. Similarly, it has been reported that Akt promotes T lymphocyte survival through enhanced expression of Bcl-x_L protein *in vivo*, without affecting its mRNA level (15). In rat pheochromocytoma (PC12) cells, Akt upregulates Bcl-2 expression through the c-AMP-response element binding protein (16), but any effect on Bcl-x_L has not been addressed. Finally, recent reports indicate that PI3-K activity can induce Bcl-x_L expression both at the mRNA and protein levels in Baf-3 cells, but this study does not implicate Akt directly (36).

Bcl-x_L has been shown to insert into the mitochondrial membrane and form ion channels (37) and to directly control mitochondrial cytochrome c release (38). In both prior studies the mitochondrial membrane potential was affected, and apoptosis was inhibited (37, 38). In some studies, Bcl-x_L has been shown to interact with caspase 9 and with apoptotic protease-activating factor (APAF-1), resulting in apoptotic inhibition (39). It has been shown in haematopoetic cells that Akt and Bcl-x_L promote interleukin 3-independent survival through distinct effects on mitochondrial physiology (40); however, it is not clear if this is the case in epithelial cells,

including mouse mammary and breast cancer cells. The mechanism by which Akt, Erk and upregulated Bcl-x_L contribute to apoptosis inhibition in our model is currently under investigation. There is a possibility, currently under exploration, that 2-fold upregulated Bcl-x_L protein could play a role in inhibition of cytochrome c release in c-Myc-overexpressing MMECs. In fibroblasts, the release of cytochrome c was reported to be involved in c-Myc-induced apoptosis (41).

Although the role of Akt in human breast cancer and MMECs is not completely understood, the role of Erk/MAPK in transformation of mouse mammary cells and in cancerous human breast tissue is firmly established in the literature (42). Here we found that EGF activates Erk and Akt within a similar time frame. In addition, we have found that, although potentially less potent than PI3-K/Akt, the MAPK/Erk pathway also delivers a survival signal in Myc83 cells. Supporting this finding, Erk has been reported to deliver a survival signal in human chondrocytes (43); in human neutrophils (44), and it was shown to cooperate with Akt in delivering survival signal to MCDK cells (26). Recently, it has been also shown that one of the ways in which the MEK/Erk pathways induce survival in keratinocytes involves expression of Bcl-x_L (28). Based on our results here, we predict the interaction of Akt and Erk in delivering survival through common targets in MMECs. One such target for both pathways could be Bcl-x_L.

In our study we have also noted a PI3-K-dependent dawnregulation of both Akt and Erk protein levels, resulting from the prolonged (24-48 hours) inhibition of PI3-K activity. Although it was previously reported that Akt is targeted by caspases during Fas-induced apoptosis of human Jurkat cells and UV-irradiated U937 cells (27), it seems that caspases are not targeting Akt in

Myc83 cells. Presently, it is not clear what are the reasons for these differences, but it could be potentially explained by the species differences. It seems that in Myc83 cells, the mechanism responsible for cleavage of both Akt and Erk may not involve caspases. When we blocked the activity of the EGFR and Erk/MAPK kinase activity, we could not detect Akt or Erk proteolysis (Figure 6c), despite the induction of apoptosis (Figure 6a and 6b). Thus, the proteolysis of Akt and Erk may be dependent on selective inhibition of PI3-K. In addition, it appears that PI3-K regulates the expression levels of Erk1 as well. The mechanism(s) of these PI3-K effects is not known at present. Our findings on Erk downregulation are consistent with a recent report, which showed that the inhibitors of PI3-K activity could block the Erk/MAPK kinase-signaling pathway (45). Also, PI3-K has been shown to control the activity of Erk/MAPK through Raf, a molecule regulating Erk function (46).

For the first time, we have demonstrated that the activated Akt could be responsible for the EGFR-regulated overexpression of Bcl-x_L in models of human breast cancer. These findings are of importance considering the unexplored role of both Akt and the Bcl-x_L in mouse mammary tumors and in human breast cancer. Recently, Akt and Bcl-x_L are both viewed as potential therapeutic targets in several human cancers and our findings could be a significant contribution. Furthermore, over-expressed Bcl-x_L protein is being considered, as a prognostic marker in a few studies of breast carcinomas where its' overexpression has been associated with advanced-stage disease.

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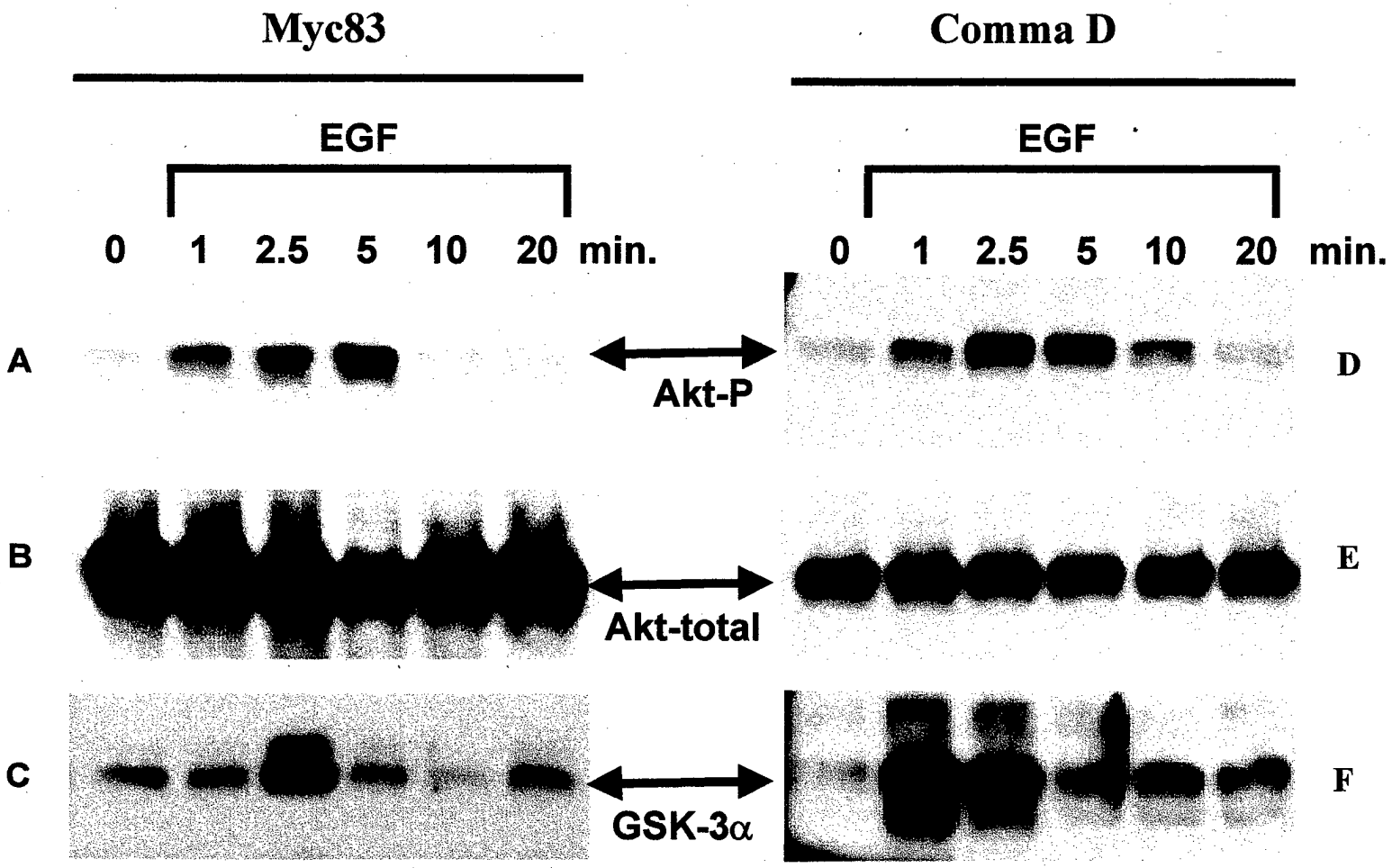
Figure 1


Myc83
Comma D
EGF
EGF
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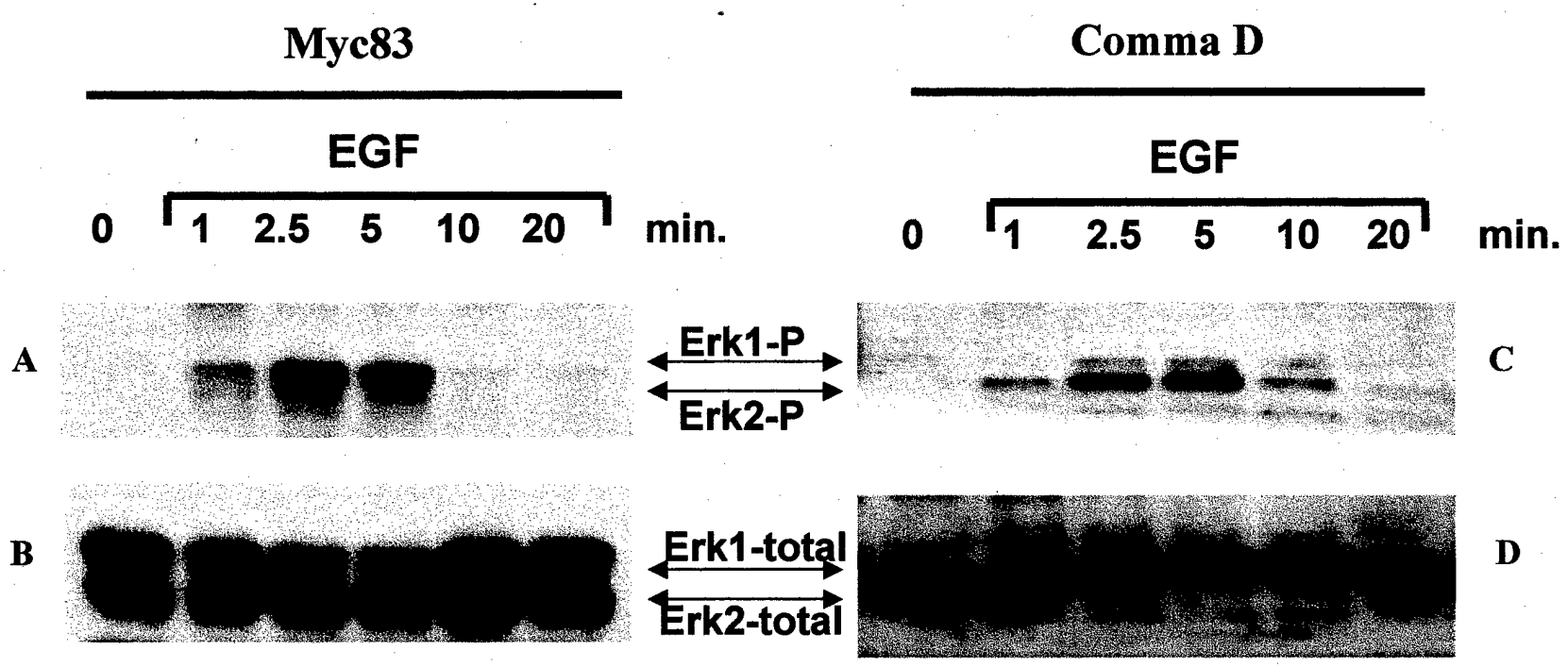
0 1 2.5 5 10 20 min.
A
Akt-P
D
B
Akt-total
E
C
GSK-3α
F

Figure 2


Myc83
Comma D
EGF
EGF
0 1 2.5 5 10 20 min.
0 1 2.5 5 10 20 min.
A
B
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Erk1-P
Erk2-P
Erk1-total
Erk2-total

Figure 3

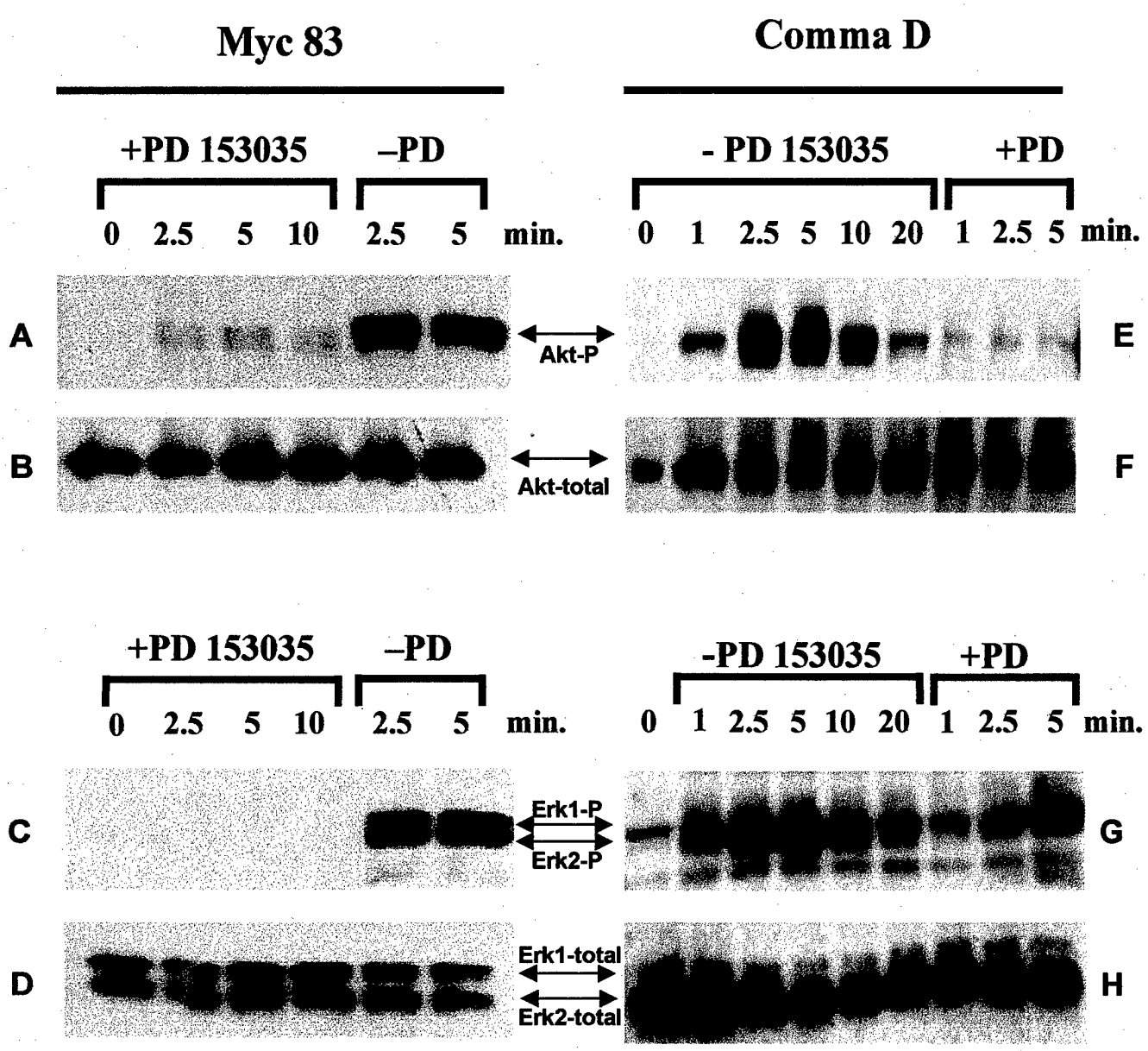

Myc 83
Comma D
+PD 153035
–PD
- PD 153035
+PD
0 2.5 5 10 2.5 5 min.
0 1 2.5 5 10 20 1 2.5 5 min.
A
Akt-P
E
B
Akt-total
F
+PD 153035
–PD
-PD 153035
+PD
0 2.5 5 10 2.5 5 min.
0 1 2.5 5 10 20 1 2.5 5 min.
C
Erk1-P
Erk2-P
G
D
Erk1-total
Erk2-total
H

Figure 4

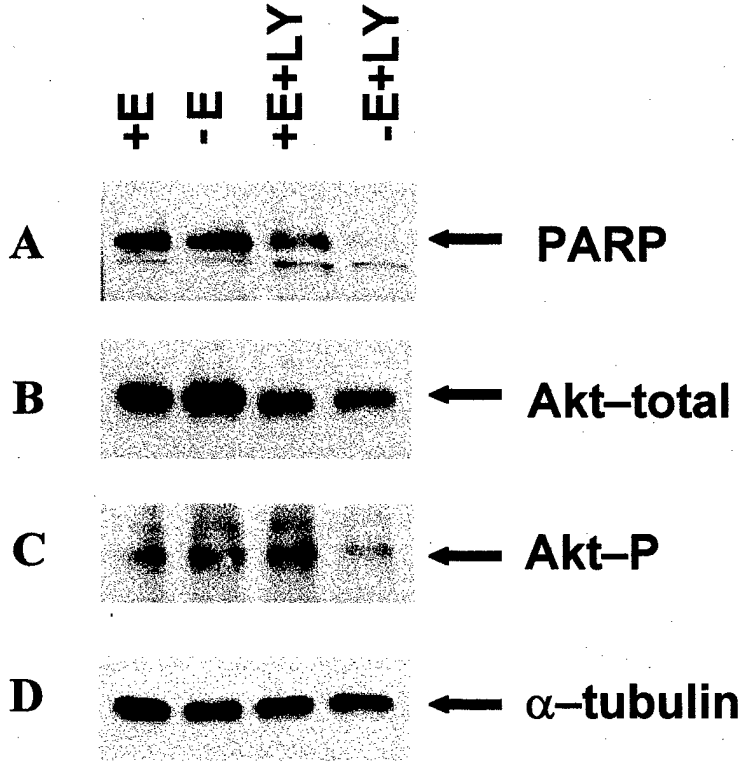


Figure 5

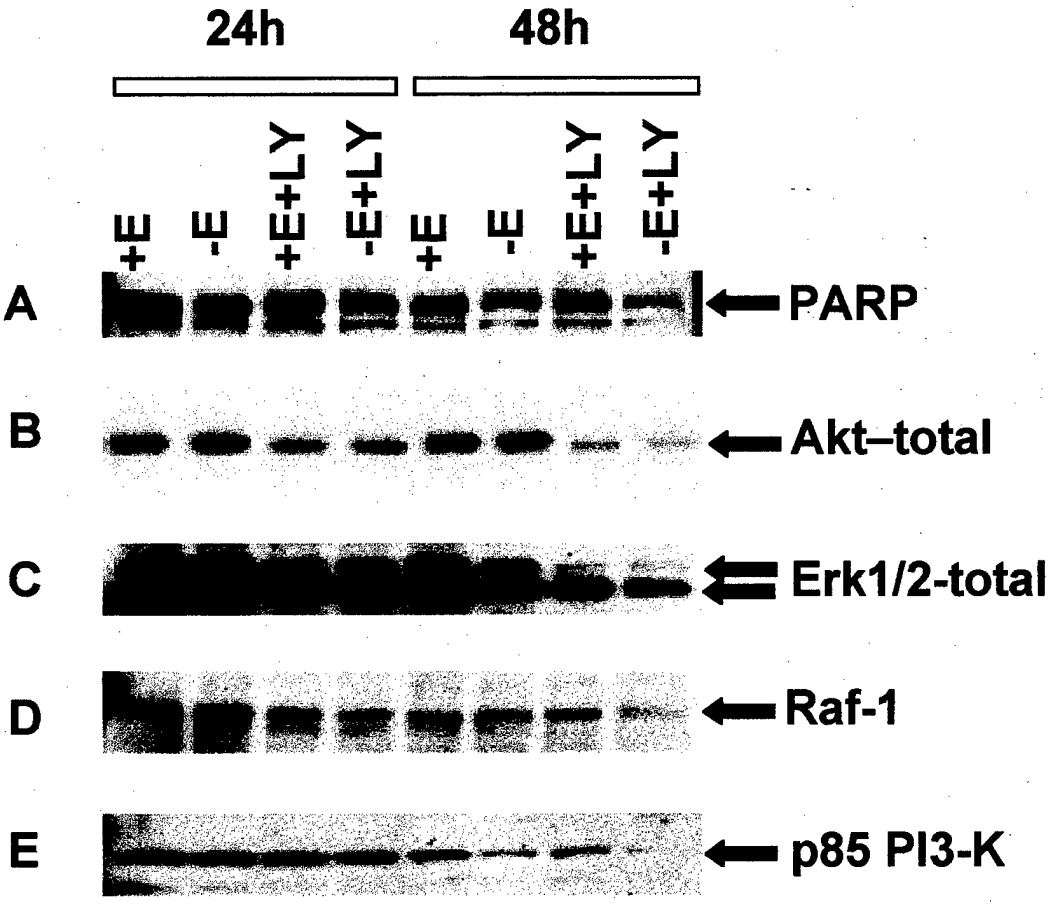
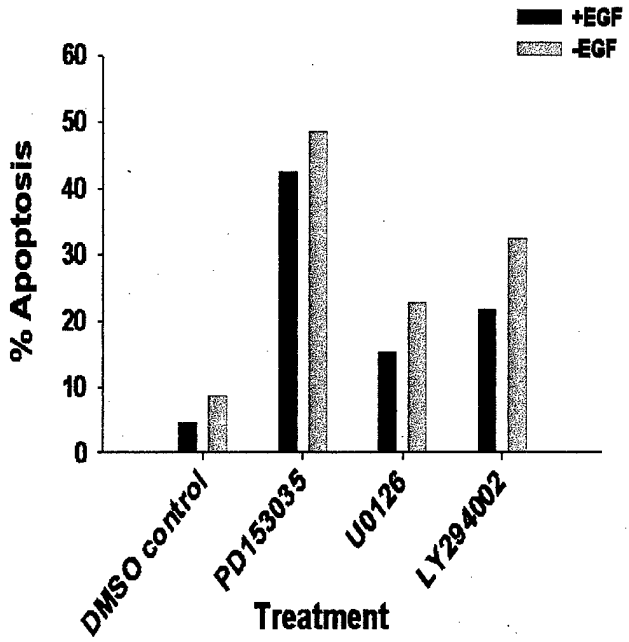

24h
48h
+E
-E
+E+LY
-E+LY
+E
-E
+E+LY
-E+LY
A
PARP
B
Akt–total
C
Erk1/2-total
D
Raf-1
E
p85 PI3-K

Figure 6

A


+EGF
-EGF
% Apoptosis
60
50
40
30
20
10
0
DMSO control
PD153035
U0126
LY294002
Treatment

B

C

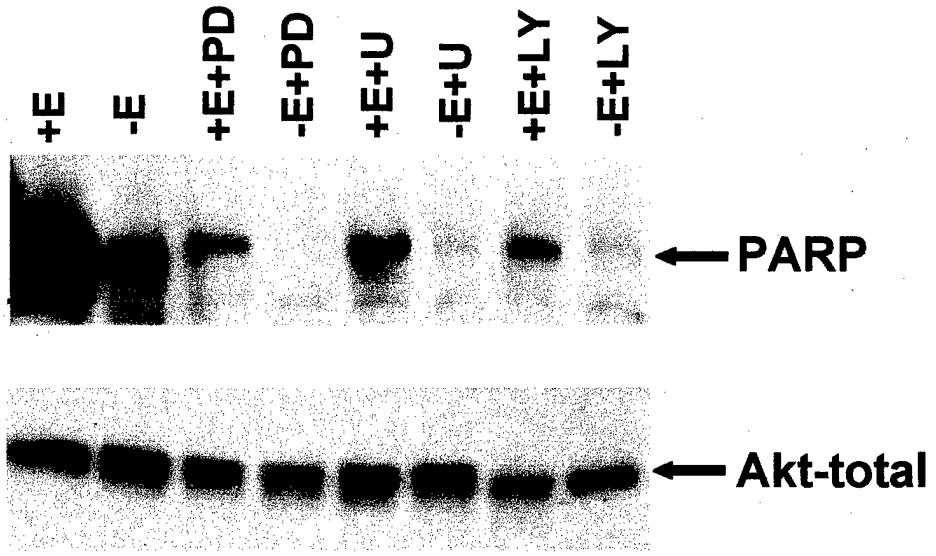
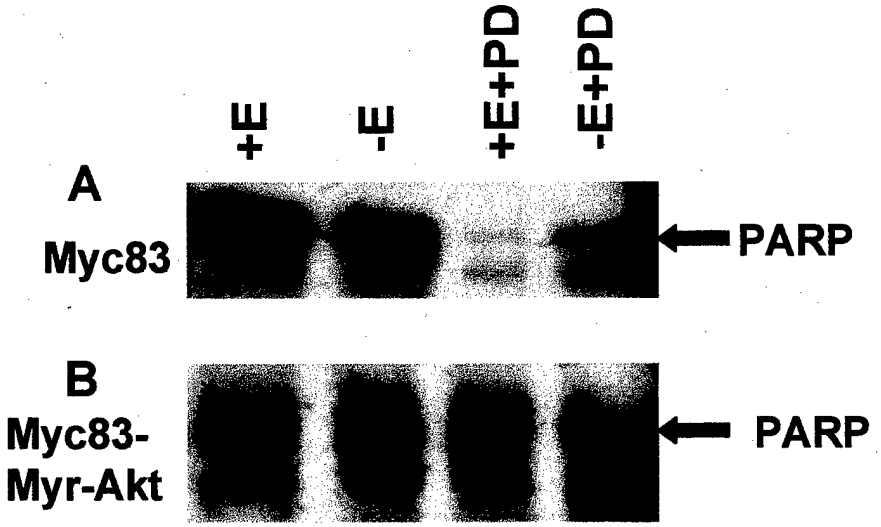

+E
-E
+E+PD
-E+PD
+E+U
-E+U
+E+LY
-E+LY
PARP
Akt-total

Figure 7


+E
-E
+E+PD
-E+PD
A
Myc83
PARP
B
Myc83-
Myr-Akt
PARP

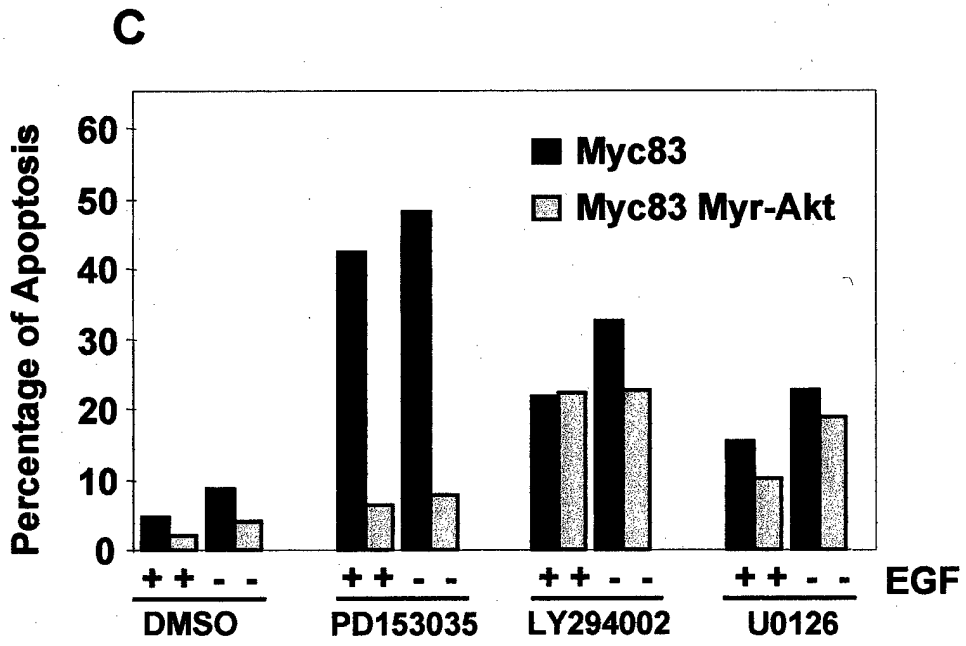

C
Percentage of Apoptosis
60
50
40
30
20
10
0
Myc83
Myc83 Myr-Akt
+ + - -
EGF
DMSO
PD153035
LY294002
U0126

Figure 8

352 **Epidermal Growth Factor Receptor Signaling Inhibits c-Myc-Induced Apoptosis through Activation of Akt, Erk and Upregulation of Bcl-xL in Mouse Mammary Carcinoma Cells**

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At present, limited information exists linking EGFR signaling pathways to cellular survival in mouse and human mammary carcinoma cells. Using a murine, c-Myc-overexpressing mammary tumor cell line (Myc83), we now show that EGFR stimulation inhibits c-Myc-induced apoptosis through Akt and Erk1/Erk2. EGF activates both Akt and Erk1/Erk2 within minutes, and this activation is completely dependent on EGFR kinase activity. Prolonged inhibition (48 hours) of EGFR kinase activity by PD153035 led to extensive cell death (50%). Inhibition of the PI-3K/Akt pathway by LY294002 and the MEK/Erk pathway by U0126 also resulted in apoptosis of over 30% and 20 % of cells respectively, confirming the importance of these two pathways for EGFR-dependent survival. In our model, apoptotic events resulting from prolonged PI3-K inhibition were paralleled by down regulation of protein expression of both Akt and Erk1/Erk2. As a further conformation of the possible survival-promoting role of Akt, we observed that constitutively activated Akt (Myr-Akt) was sufficient to promote survival of apoptotic c-Myc-overexpressing cells following inhibition of the EGFR tyrosine kinase activity. In addressing possible downstream mediators of Akt-induced survival, we observed significant upregulation (4-fold) of the anti-apoptotic protein Bcl-xL in Myc83-Myr-Akt cells, suggesting for the first time that this protein may be a target of Akt and may be involved in Akt-mediated survival. These data support the need for further study of Akt and its downstream targets in cooperation with Erk for inhibition of c-Myc-induced apoptosis in mammary tumorigenesis. (This work was supported by the NIH RO1 CA72460)

THE ROLE OF C-MYC OVEREXPRESSION IN SENSITIZATION OF MAMMARY EPITHELIAL CELLS TO APOPTOSIS

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The c-myc oncogene is thought to play an important role(s) in the onset and progression of breast cancer, where it is amplified in approximately 15% and overexpressed in over 60% of human breast cancers. Depending upon the avaliability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through as yet poorly defined mechanisms. Mammary tumors from c-myc transgenic mice are characterized by a long tumor latency and an elevated apoptotic index. Currently the molecular mechanism by which c-Myc sensitizes cells to apoptosis is not fully understood. Also unclear is the exact type of stimuli to which c-Myc-overexpressing cells respond to apoptotically, and what environmental conditions in c-Myc-overexpressing mammay epithelium promote apoptosis versus proliferation and transformation. Currently, the molecular mechanisms by which c-Myc has been shown to mediate apoptosis are the Fas/Fas ligand pathway, p53 and mitochondrial-dependent release of cytochrome C. Previously it has been demonstrated in our laboratory that a mammary carcinoma cell line (Myc83), derived from MMTV-c-myc transgenic mouse, are sensitive to EGF withdrawl and undergo extensive apoptosis upon inhibition of EGFR signaling. Apoptosis of Myc83 under these conditions was accompanied by the up-regulation of the death receptor Fas, but not of its ligand FasL, as previously thought. This apoptosis was preceeded by the activation of the pro-apoptotic stress related MAPK, p38.

The present project studied c-Myc's sensitization to apoptosis of both Myc83 cells, and a non-tumorgenic immortalized mouse mammary epithelial line overexpressing c-Myc (FSK7-Myc) compared to the control FSK7-LXSN cells. Here we show that growth factor withdrawl/blockade, rFasL, and other apoptotic stimuli resulted in apoptosis in Myc83 and FSK7-Myc cells but not in the control FSK7-LXSN cells, suggesting that c-Myc overexpression in mammary epithelial sensitizes them to apoptotic stimuli. However, evaluation of the mechanism responsible for apoptosis induced by growth factor withdrawl in Myc-expressing cells revealed a lack of a role for both endogenous Fas/FasL and p38-MAPK in mediating this c-Myc sensitization to apoptosis.

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