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Increased Expression of Mcl-1 Is Responsible for the Blockage of TRAIL-Induced Apoptosis Mediated by EGF/ErbB1 Signaling Pathway

Elizabeth S. Henson, Erika M. Gibson, Jacylyn Villanueva, Nicolle A. Bristow, Neil Haney, and Spencer B. Gibson*

Manitoba Institute of Cell Biology, University of Manitoba, 675 McDermot Ave., Winnipeg, Manitoba, R3E 0V9

Abstract Epidermal growth factor (EGF) protects against death receptor induced apoptosis in epithelial cells. Herein, we demonstrate that EGF protection against tumor necrosis factor related apoptosis-inducing ligand (TRAIL) induced apoptosis is mediated by increased expression of the Bcl-2 family member myeloid cell leukemia 1 (Mcl-1). EGF increased the mRNA and protein levels of Mcl-1. Furthermore, expression of ErbB1 alone or in combination with ErbB2 in NIH3T3 cells up-regulates Mcl-1 following EGF treatment. In addition, up-regulation of Mcl-1 by EGF is mediated through AKT and NFκB activation since kinase inactive AKT and ΔIκB effectively blocks this up-regulation. NFκB was also critical for the ability of EGF to prevent TRAIL induced apoptosis as a dominant negative IκB (ΔIκB) blocked NFκB activation, and relieved EGF protection against TRAIL mediated mitochondrial cytochrome-*c* release and apoptosis. Finally, anti-sense oligonucleotides directed against Mcl-1 effectively reduced the protein levels of Mcl-1 and blocked EGF protection against TRAIL induced mitochondrial cytochrome-*c* release and apoptosis. Taken together, EGF signaling leads to increased Mcl-1 expression that is required for blockage of TRAIL induced apoptosis. J. Cell. Biochem. 89: 1177–1192, 2003.

Key words: apoptosis; survival; death receptors; EGF; Mcl-1; cancer

Epidermal growth factor (EGF) binds to the EGF receptor family (ErbB receptors) [Cohen, 1997]. There are four members of the ErbB receptor family, ErbB1 (EGFR, HER1), ErbB2 (HER2, neu), ErbB3 (HER3), and ErbB4 (HER4) [Olayioye et al., 2000]. In the presence of extracellular ligands, ErbB family members form both homodimers and heterodimers. EGF binds to ErbB1 homodimers as well as ErbB1:ErbB2, ErbB1:ErbB3, or ErbB1:ErbB4 heterodimers [Pinkas-Kramarski et al., 1997]. Activation of ErbB1 and its heterodimers leads to cellular proliferation or differentiation dependent on the cellular circumstances as well as cellular protection against apoptosis [Olayioye et al., 2000]. Up-regulation of these receptors was found in lung, ovarian, and breast cancer contributing to cell survival [Pinkas-Kramarski et al., 1997; Orr et al., 2000]. The identity of the ErbB receptor homodimers or heterdimers responsible for EGF protection against apoptosis are, however, unknown.

Myeloid cell leukemia 1 (Mcl-1) is a Bcl-2 family member that is expressed in wide variety of cells including epithelial cells [Backus et al., 2001]. Its expression is increased after treatment with growth factors, and cytokines including macrophage colony stimulating factor, interleukin 6 and interleukin 3 [Lin et al., 2001; Puthier et al., 2001]. Similar to other Bcl-2 family members, overexpression of Mcl-1 protects cells from apoptosis through blockage of cytochrome-*c* release from the mitochondria [Wang and Studzinski, 1997]. In addition, Mcl-1 expression is increased in several cancers including ovarian cancer, and leukemia contributing to cell survival [Derenne et al., 2002; Rassidakis et al., 2002].

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*Correspondence to: Dr. Spencer B. Gibson, Assistant Professor, Manitoba Institute of Cell Biology, University of Manitoba, 675 McDermot Ave., Winnipeg, Manitoba, R3E 0V9. E-mail: gibsonsb@cc.umanitoba.ca

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Tumor necrosis factor related apoptosis-inducing ligand (TRAIL) belongs to a subfamily of death receptor ligands of the tumor necrosis factor (TNF) super-family [Ashkenazi and Dixit, 1999]. TRAIL binds to death receptor 4 (DR4) and death receptor 5 (DR5) initiating the recruitment of the adaptor protein Fas associated death domain (FADD) and caspase 8 [Ashkenazi and Dixit, 1999]. This leads to further downstream caspases being activated and apoptosis. Caspase 8 also directly cleaves the Bcl-2 family member BID. Truncated BID (tBID) translocates to the mitochondria contributing to the release of cytochrome-*c* [Ashkenazi and Dixit, 1999]. Cytochrome-*c* binds to Apaf1 and caspase 9 in the apoptosome, activating caspase 9 leading to cell death [Bernardi et al., 1999].

TRAIL unlike other members of the TNF family is able to selectively induce apoptosis in tumor cells without affecting surrounding normal tissue [Ashkenazi and Dixit, 1999; Walczak et al., 1999]. In mice, TRAIL seems to be nontoxic and in combination with chemotherapeutic drugs eliminates human breast tumors grown in SCID mice [Walczak et al., 1999]. Thus, TRAIL is a potential treatment for cancers and an understanding of the mechanisms regulating its ability to induce cell death in tumors cells is of significant importance in designing new therapeutic approaches.

Herein, we have shown that EGF binding to ErbB1 is sufficient to prevent TRAIL induced apoptosis. This protection is mediated by upregulation of Mcl-1. We also determined that EGF activation of transcription factor NFκB increases Mcl-1 expression. Thus, EGF induced Mcl-1 expression could be responsible for EGF protection against TRAIL induced apoptosis.

MATERIALS AND METHODS

Materials

EGF was purchased from Sigma-Aldrich Canada Ltd., Ontario, Canada and dissolved in 10 mM acetic acid containing 0.1% BSA. Heregulin was purchased from R&D Systems Inc., Minneapolis, MN and reconstituted in phosphate buffered saline (PBS) containing 0.1% BSA. Anti-cytochrome-*c* was purchased from PharMingen, BD Biosystems, Ontario, Canada and anti-mouse Rhodamine Red was purchased from Molecular Probes, Eugene, OR. Anti-Mcl-1 antibodies and anti-Bcl-$x_{L/S}$ antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA and anti-actin antibodies was purchased from Sigma.

Cell Culture

All cell lines were maintained in a humidified 5% CO_2 environment with appropriate media. Human embryonic kidney 293 (HEK293) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U of penicillin per ml, 100 μg of streptomycin (Gibco Laboratories, Grand Island, NY) per ml, and 10% bovine calf serum. HEK293 cells stably expressing vector alone (pcDNA3), kinase inactive AKT (AKTK-M), and dominant negative IκB (ΔIκB) were maintained under selection with 1.5 mg of G418 (Gibco Laboratories) per ml. All NIH3T3 cell lines (kind gift from Dr. N. Haynes, Friedrich Miescher Institute) were maintained in DMEM supplemented with 100 U of penicillin per ml, 100 μg of streptomycin (Gibco Laboratories) per ml, and 10% fetal bovine serum. MDA MB 231 cells were maintained in DMEM supplemented with 100 U of penicillin per ml, 100 μg of streptomycin (Gibco Laboratories) per ml, 10% fetal bovine serum, 10% non-essential amino acids (Gibco Laboratories), and 40 ng/ml of insulin (Gibco Laboratories).

Isolation of his-Tagged Soluble TRAIL

E. coli (BL21), containing the pET plasmid with 101–281 a.a. soluble TRAIL cDNA (kind gift from Dr. Shu, National Jewish Medical and Research Center), were grown overnight under selection (50 μg/ml ampicillin) and diluted 1 in 2. The bacteria was treated with 1 mM IPTG for 2 h and lysed in lysis buffer (10 mM Tris pH 8.0, 100 mM NaCl, 0.1% NP-40, 5 mM imidizole, and 2 mM PMSF). The lysate was sonicated 4× for 15 s and centrifuged at 8,000*g* for 30 min. Nickel sepharose beads (500 μl) were added to the lysate and incubated for 2 h at 4°C. The beads were then washed in wash buffer (10 mM Tris pH 8.0, 100 mM NaCl, 0.1% NP-40, and 5 mM imidizole) three times and eluted with elution buffer (10 mM Tris pH 8.0, 100 mM NaCl, 0.1% NP-40, and 100–300 mM imidizole). The isolated TRAIL was used to treat cells as indicated.

Treatment of Cells With EGF and TRAIL

Where indicated, cells were pre-treated with 1 μg/ml of EGF for 1 h before the addition of TRAIL. We used 1 mg/ml EGF to treat cells

since it gave the strongest anti-apoptotic response against TRAIL induced apoptosis. We, however, found that doses as low as 100 ng/ml EGF gave protection against TRAIL induced apoptosis. TRAIL was added at a concentration of 1 μg/ml for all HEK293, NIH3T3 parental, and NE1 (expressing ErbB1) cell lines, 2 μg/ml was added to the NE1/2 (expressing both ErbB1 and ErbB2) cell line. This dose of TRAIL induces significant levels of apoptosis after 24 h in the cell lines tested.

Immunohistochemistry

HEK 293 cells were grown on glass coverslips overnight and treated with EGF alone, TRAIL alone, and in combination. After 24 h of treatment, the coverslips were removed and fixed overnight in 3.7% formaldehyde in 1× PBS. The next morning, the slips were washed twice for 5 min with 500 μl of 0.1% NP-40 in 1× PBS. Mouse anti-cytochrome-*c* was diluted 1 in 500 in 10% fetal bovine serum, 0.1% NP-40 in 1× PBS, and the slips were incubated 1.5 h. The coverslips were then washed twice in 500 μl 0.1% NP-40 in 1× PBS. Secondary antibody anti-mouse Rhodamine Red at a dilution of 1 in 500 in 10% fetal bovine serum, 0.1% NP-40 in 1× PBS was added, and the slips incubated 1.5 h in the dark. After the incubation period was over, the slips were washed with 0.1% NP-40 in 1× PBS and then incubated for 6 min in 1 in 2,000 dilution of 20 mM Hoescht stain (Sigma) in 0.1% NP-40 in 1× PBS in the dark. Coverslips were mounted onto slides with 4 μl Fluoroguard anti-fade reagent (BioRad Laboratories, Ontario, Canada). No fewer than 200 cells were counted per sample. Fluorescence was visualized and captured using a Zeiss Axiphot microscope with a cooled charge-coupled device camera.

Measurement of Apoptosis

Cells were resuspended by gentle vortexing in 100 μl of medium and 2 μl of acridine orange (100 μg/ml) and ethiduim bromide (100 μg/ml) in PBS was added. Ten microliters of this solution was added to a slide and a coverslip applied over the cells. The slide was viewed on an Olympus CK40 fluorescent microscope using a fluorescein filter set for the detection of condensed DNA in apoptotic cells. The condensed DNA was defined by intense local staining of DNA in the nucleus compared to the diffuse staining of the DNA in normal cells. The amount of apoptosis was determined from the cells containing normal DNA staining compared to the cells with condensed DNA and morphological changes consistent with apoptosis.

RNAse Protection Assay

A RiboQuant Multi-Probe RNase Protection Assay System (PharMingen) was used as per the manufacturer's instructions. HEK293 vector alone cells were treated with EGF (1 μg/ml) for 1 h, or left untreated as a control. RNA was harvested using RNAzol^{TM}B, and 20 μg of RNA was hybridized with the hAPO-2b template set (PharMingen). This probe set contains templates for Bclw, Bcl-$x_{(L,S)}$, Bfl1, Bid, Bik, Bak, Bax, Bcl-2, Mcl-1, L32, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The antisense RNA probes were synthesized by T7 RNA polymerase and labeled using [α^{32}-P] UTP. Free probe and other single stranded RNA were digested with RNAse, and the remaining protected dsRNA was run out on a polyacrylamide gel. An aliquot of the probe was run in the first lane. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the samples. Analysis of the signal intensity was done on a Storm Phosphoimager (Amersham-Pharmacia, Piscataway, NJ).

Immunoblots

Cells were lysed in Nonidet P-40 lysis buffer (50 mM HEPES pH 7.25, 150 mM NaCl, 50 μM $ZnCl_2$, 50 μM NaF, 2 mM EDTA, 1 mM Na vanadate, 1.0% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation 8,000*g* for 5 min and protein concentration was determined by a Bradford assay. Five hundred micrograms of cell lysate was subject to SDS–polyacrylamide electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with 0.15% Tween-20 (TBST) and 5% milk. Blots were incubated with the appropriate antibody concentration overnight, washed three times in 1× TBST and incubated 1 h with the appropriate secondary antibody conjugated with alkaline phosphatase. Blots were visualized on X-ray film with enhanced chemiluminence reagents (NEN Dupont, Derkin Ever, Boston, MA).

NFκB Luciferase Assay

NIH3T3 cells were transiently transfected with 1 μg of the NFκB luciferase reporter gene

and 0.2 μg of the β-gal reporter gene using the GenePorter transfection reagent (Gene Therapy Systems, San Diego, CA) as per manufactures instructions. Transfection efficiency was determined by an enzymatic assay for β-gal (Promega, Madison, WI) read on an ELISA reader. Eighteen hours post-transfection cells were treated with EGF and lysed in 5× lysis buffer for luciferase (Promega). Luciferase activity was measured after the addition of 100 μl of luciferin reagent (Promega) in a microtiter plate luminometer (L-max, Molecular Devices, Sunnyvale, CA).

Transfection of Anti-Sense Oligonucleotides Against Mcl-1

The oligonucleotides were obtained from Sigma Genosys and were all HPLC purified. All oligonucleotides were designed with phophorothioate nucleotides at each end represented by the lower case letter in the sequence. Anti-sense (catccCAGCCTCTTtgtttA) and sense (atttgTTTCTCCGAccctaC) sequences were previously published [Moulding et al., 2000]. Scramble oligonucleotide (tacgtTCGTTTCCAcctcaT) was a combination of the same nucleotides as in the anti-sense sequence but in a different order. The scrambled sequence was run through BLAST SEARCH, and no significant homologies exist with other genes. HEK293 cells were plated at a density of 0.5×10^6 cells in 6-well dishes (Corning Inc., Life Sciences, Acton, MA). Twenty-four hours after plating the cells were washed once with DMEM, and DMEM was then added to each well. Each oligonucleotide (160 nM) was diluted in Opti-MEM (Invitrogen Technologies, Carlsbad, CA), and 2 μl of Oligofectamine Reagent (Invitrogen) were each diluted in Opti-MEM. The solutions were incubated at room temperature for 5 min before the Oligofectamine solution was added drop by drop to the oligonucleotide solution. This final solution was incubated 20 min at room temperature and then added to the cells. The cells were then incubated for 4 h, and a 30% BCS DMEM solution was added. At this time 1 μg/ml of EGF was added and the cells harvested after 0.5, 1, and 2 h.

RESULTS

EGF Binding to ErbB1 Is Sufficient to Protect Cells Against TRAIL Induced Apoptosis

EGF binds to ErbB1 alone or in combination with ErbB2, ErbB3, or ErbB4 [Cohen et al., 1999; Klambt, 2000]. The ErbB receptors responsible for EGF protection against TRAIL-induced apoptosis are unknown. In NIH3T3 cells expressing specific EGF receptors, the ability of these EGF receptors to protect against TRAIL induced apoptosis was determined. NIH3T3 cells specifically selected for their lack of expression of EGF receptors (parental), expressing human ErbB1 only (NE1) or expressing human ErbB1 and ErbB2 (NE1/2) [Olayioye et al., 2000] were untreated, treated with 1 μg/ml EGF alone, 1 μg/ml TRAIL alone, or in combination. Parental cells untreated or treated with EGF had very low levels of apoptosis (5.2 and 3.3%, respectively). The amount of apoptosis was similar in cells treated with TRAIL alone (29.1%) or in combination with EGF (24.4, Fig. 1). When the ErbB1 receptor is expressed alone in NIH3T3 cells, untreated and EGF treated cells had low levels of apoptosis (4.4 and 4.3%, respectively). The level of apoptosis in cells treated with TRAIL alone was 24.9% similar to parental cells but in combination with EGF the amount of apoptosis was significantly reduced (13.4%, Fig. 1). In cells expressing both ErbB1 and ErbB2 in combination, the levels of apoptosis in untreated and EGF treated cells were comparable to parental and ErbB1 cells lines at 4.5 and 4.4%. However, these cells were more resistant to TRAIL induced apoptosis, since doubling the concentration of TRAIL (2 vs. 1 μg/ml) gave only 18.8% apoptosis (Fig. 1). EGF treatment still protected against TRAIL mediated apoptosis even at the increased concentrations of TRAIL (8.9%). This suggests that ErbB1 alone is sufficient to protect cells from TRAIL induced apoptosis and in combination with ErbB2 is capable of mediating EGF protection against apoptosis.

EGF Increases the Expression of the Anti-Apoptotic Bcl-2 Family Member Mcl-1

Bcl-2 family members regulate apoptosis and EGF protects cells against apoptosis. To determine if EGF increased Bcl-2 family members, HEK 293 cells were treated with EGF over a time course and mRNA levels of both pro- and anti-apoptotic Bcl-2 family members were determined as described in Materials and Methods. Bcl-2 mRNA levels were detectable at low levels after treated with EGF. Bcl-w, Bcl-x_L, Bid, Bfl, Bak, and Bax mRNA levels were not significantly increased following EGF treat-

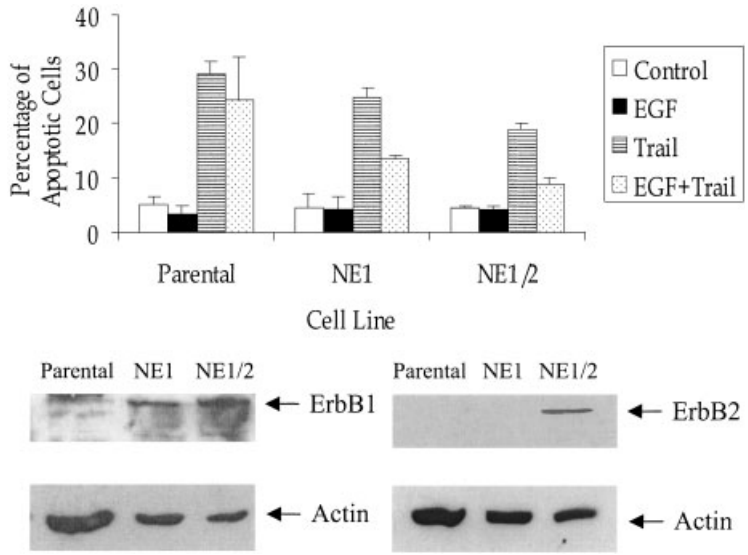

Fig. 1. Epidermal growth factor (EGF) binding to ErbB1 alone and in combination with ErbB2 protects cells against tumor necrosis factor related apoptosis-inducing ligand (TRAIL) induced apoptosis. NIH3T3 cells were pre-incubated with or without 1 μg/ml of EGF for 1 h before 1 μg/ml of soluble his-TRAIL (parental and NE1) or 2 μg/ml of soluble his-TRAIL (NE1/2) was added to the culture medium. As a positive control, cells were treated with TRAIL alone. After 24 h, the amount of apoptosis was quantified by acridine orange staining of DNA as described in Materials and Methods. Error bars represent the standard deviation of three separate experiments. The lower panels indicate the relative expression of ErbB1 and ErbB2 in this cell lines.

ment for 2 h (Fig. 2A). It appears the Bak mRNA levels are decreased following EGF treatment at 2 h (Fig. 2A) but over a time course Bak mRNA levels remained unchanged (Fig. 2B). However, the anti-apoptotic Bcl-2 family member Mcl-1 was increased 4.9-fold at 1 h, and 3.5-fold after 2 h (Fig. 2B). In addition to mRNA levels for Mcl-1, protein levels of Mcl-1 were also increased following EGF treatment (data not shown).

NIH3T3 Cells Expressing ErbB Receptors Selectively Increase Mcl-1 Expression

In addition to HEK293 cells, the ability of EGF to increase Mcl-1 in NIH3T3 cells expressing ErbB receptors was determined. In NIH3T3 parental cells that do not express EGF receptors, there is a very low level of Mcl-1 expression as detected by anti-Mcl-1 antibodies and the levels fails to increase following EGF treatment. In contrast, NIH3T3 cells expressing ErbB1 (NE1) showed detectable levels of Mcl-1 only after EGF stimulation while NIH 3T3 cells expressing ErbB1 and ErbB2 (NE1/2) showed detectable levels of Mcl-1 without EGF treatment and Mcl-1 levels increased following EGF treatment over a 4 h time course (Fig. 3). In NIH3T3 cells expressing ErbB1 or ErbB1:ErbB2, EGF increased Mcl-1 peaking at 2 h (Fig. 3). Mcl-1 expression was still present at 24 h after EGF treatment albeit at lower levels (data not shown). Bcl-x_L expression failed to increase in each of these cells following EGF treatment but was expressed in all NIH3T3 cell lines at equal levels (data not shown). This indicates that expression of ErbB1/2 receptors on the cell surface is sufficient to increase basal Mcl-1 expression and following EGF treatment, Mcl-1 protein expression in NIH3T3 expressing ErbB1 and ErbB1:ErbB2 is further increased.

Anti-Sense Oligonucleotides Against Mcl-1 Blocks EGF Mediated Up-Regulation of Mcl-1 Expression and Inhibits the Protective Effects of EGF on TRAIL Induced Apoptosis

Anti-sense oligonucleotides against Mcl-1 were previously shown to be effective at specifically reducing Mcl-1 protein levels in the myeloid cell line U937 [Moulding et al., 2000]. We used these anti-sense oligonucleotides to determine if Mcl-1 levels in HEK293 cells can be reduced and if anti-sense oligonucleotides against Mcl-1 blocks EGF mediated up-regulation of Mcl-1 expression. HEK293 cells were transfected with anti-sense oligonucleotides against Mcl-1 as described in Materials and Methods and treated with EGF. The levels of Mcl-1 protein failed to increase following EGF treatment (Fig. 4A). As negative controls, both scrambled and sense oligonucleotides were transfected into HEK293 cells and stimulated

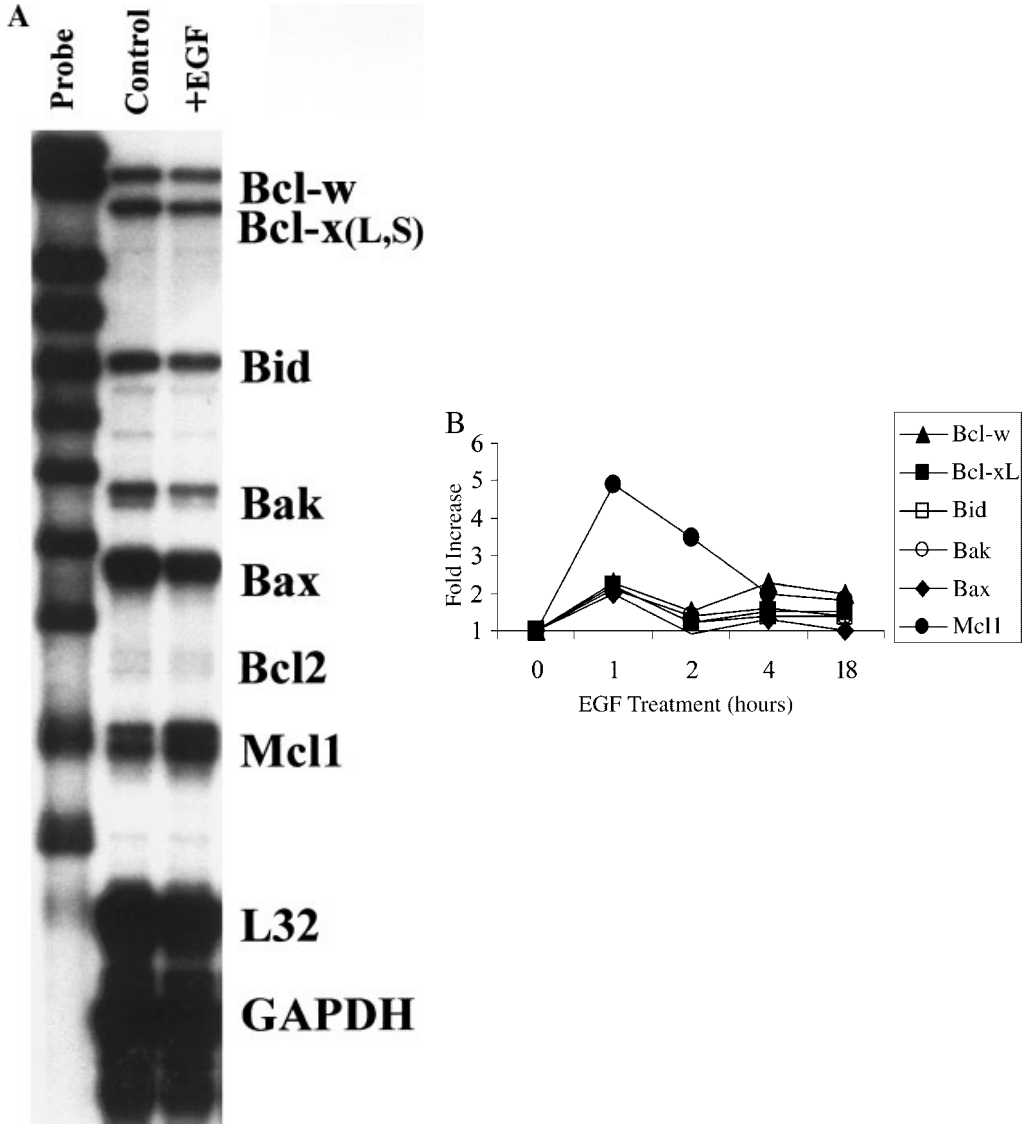

Fig. 2. EGF treatment up-regulates the anti-apoptotic Bcl-2 family member myeloid cell leukemia 1 (Mcl-1). **A**: mRNA levels for Bcl-2 family members were determined by RNAse protection assay. HEK293 cells were treated with EGF (1 μg/ml) for a time course of over 18 h, or left untreated as a control. RNA was harvested using RNAzol^{TM}B at various times, and 20 μg of RNA was hybridized with the hAPO-2b template set(PharMingen, BD Biosystems, San Jose, CA). Free probe and other single stranded RNA were digested with RNase, and the remaining protected dsRNA was run out on a polyacrylamide gel and exposed on film. An aliquot of the probe was run in the first lane. The housekeeping gene *GAPDH* was used to normalize the samples. This figure represents mRNA levels at 2 h following EGF treatment. **B**: Analysis of the signal intensity was done on a Storm Phosphoimager (Amersham-Pharmacia, Piscataway, NJ). Fold increase in mRNA levels were normalized to GAPDH over a 18 h time course. This is representative of three independent experiments.

with EGF. Mcl-1 levels increased following EGF treatment (Fig. 4A) to levels similar to untransfected HEK293 cells (data not shown). In contrast, Bcl-x_L protein levels remained unchanged under all conditions tested (data not shown). Since EGF effectively protects cells against TRAIL induced apoptosis, we determined if reduction in Mcl-1 levels eliminates EGF survival response against TRAIL induced apoptosis. HEK293 cells were treated with TRAIL, EGF, or in combination. TRAIL alone induced apoptosis (28%) while in combination with EGF the amount of apoptosis was reduced to 13% that was comparable to the amount of apoptosis in untreated and EGF alone treated cells (8.2 and 7.1%, respectively). This showed that EGF protects HEK293 cells against TRAIL induced apoptosis (Fig. 4B). TRAIL and

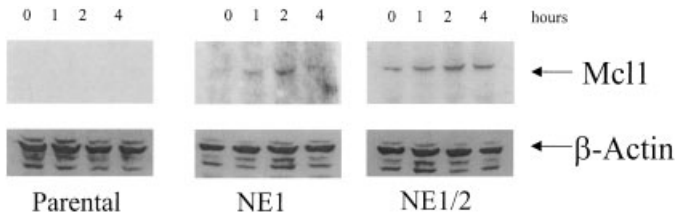

Fig. 3. Mcl-1 protein levels in NIH3T3 cells expressing ErbB receptors NIH3T3 parental, NE1 and NE1/2 cells (as described in this figure) were treated with 1 μg/ml of EGF over a 4 h time course. The cells were then lysed in lysis buffer and Western blotted for Mcl-1, and Bcl-x_L as described in Materials and Methods. The blots were stripped and reprobed with anti-β-actin to show equal loading of protein. These experiments were repeated three independent times.

EGF were then treated to cells in the presence of anti-sense, scrambled, or sense oligonucleotides. Only anti-sense restored the ability of TRAIL to induce apoptosis in the presence of EGF with 26% apoptotic cells after 36 h while sense and scrambled oligonucleotides fails to inhibit EGF protection against TRAIL induced apoptosis (Fig. 4B). Furthermore anti-sense, sense, and scrambled oligonucleotides failed to significantly alter TRAIL induced apoptosis in the absence of EGF (Fig. 4B). This indicates that Mcl-1 plays a significant role in EGF protection against TRAIL induced apoptosis.

We have previously shown that EGF mediated by AKT activation effectively blocks TRAIL induced mitochondrial cytochrome-*c* release. To determine whether EGF blockage of TRAIL induced mitochrondrial cytochrome-*c* release is regulated by Mcl-1 induction, we immunofluorescently stained HEK293 cells with antibodies against cytochrome-*c* in the presence or absence of anti-sense oligonucleotides against Mcl-1. The release of cytochrome-*c* from the mitochondria was then measured as described in Materials and Methods. TRAIL mediated release cytochrome-*c* from the mitochondria was effectively blocked by EGF treatment (44% with TRAIL alone compared to 25% with TRAIL in combination with EGF, Fig. 4C). This represents a 56% reduction in mitochondrial cytochrome-*c* release taking into account background cytochrome-*c* release due to spontaneous apoptosis after EGF treatment. In the presence of anti-sense oligonucleotides against Mcl-1, 42% of cytochrome-*c* was released from the mitochondria following TRAIL treatment alone and 36% of cytochrome-*c* was released from the mitochondria in the presences of EGF (Fig. 4C). This suggests that EGF protection against mitochondrial cytochrome-*c* release involves Mcl-1. As negative controls, both sense and scrambled oligonucleotides failed to alter the amount of cytochrome-*c* released from the mitochondria induced by TRAIL in the presence or absence of EGF (data not shown). Taken together, this indicates that the protective effects of EGF are mediated by up-regulation of Mcl-1.

Inhibition of EGF Mediated AKT and NFκB Activation Blocks EGF Up-Regulation of Mcl-1

Since EGF activates both AKT and NFκB and both are involved in up-regulation of anti-apoptotic genes, we determined if AKT and NFκB are involved in EGF up-regulation of Mcl-1. In HEK293 expressing ΔIκB and AKT-KM, Mcl-1 protein levels remained unchanged following EGF treatment over a 4 or 8 h time course, respectively. This was not due to differences in protein concentrations since both Bcl-x_L and β-actin remained unchanged in both ΔIκB and AKT-KM expressing cells (Fig. 5A,B). In addition, p50 subunit of NFκB was overexpressed in HEK293 cells causing the basal NFκB transcriptional activity to increase by 10-fold that resulted in increased expression of Mcl-1 in two independent clones (data not shown). Thus, Mcl-1 expression could be a target of EGF mediated AKT and NFκB activation.

EGF Protection Against TRAIL Induced Mitochondrial Cytochrome-*c* Release and Apoptosis Requires NFκB Activation

We have previously demonstrated that the ability of EGF to protect cells against TRAIL induced apoptosis is mediated by AKT [Gibson et al., 2002]. Both EGF and AKT activate NFκB [Habib et al., 2001]. To determine if the ability of EGF to protect cells from TRAIL-induced apoptosis requires functional NFκB, cells expressing ΔIκB (effectively blocks NFκB tran-

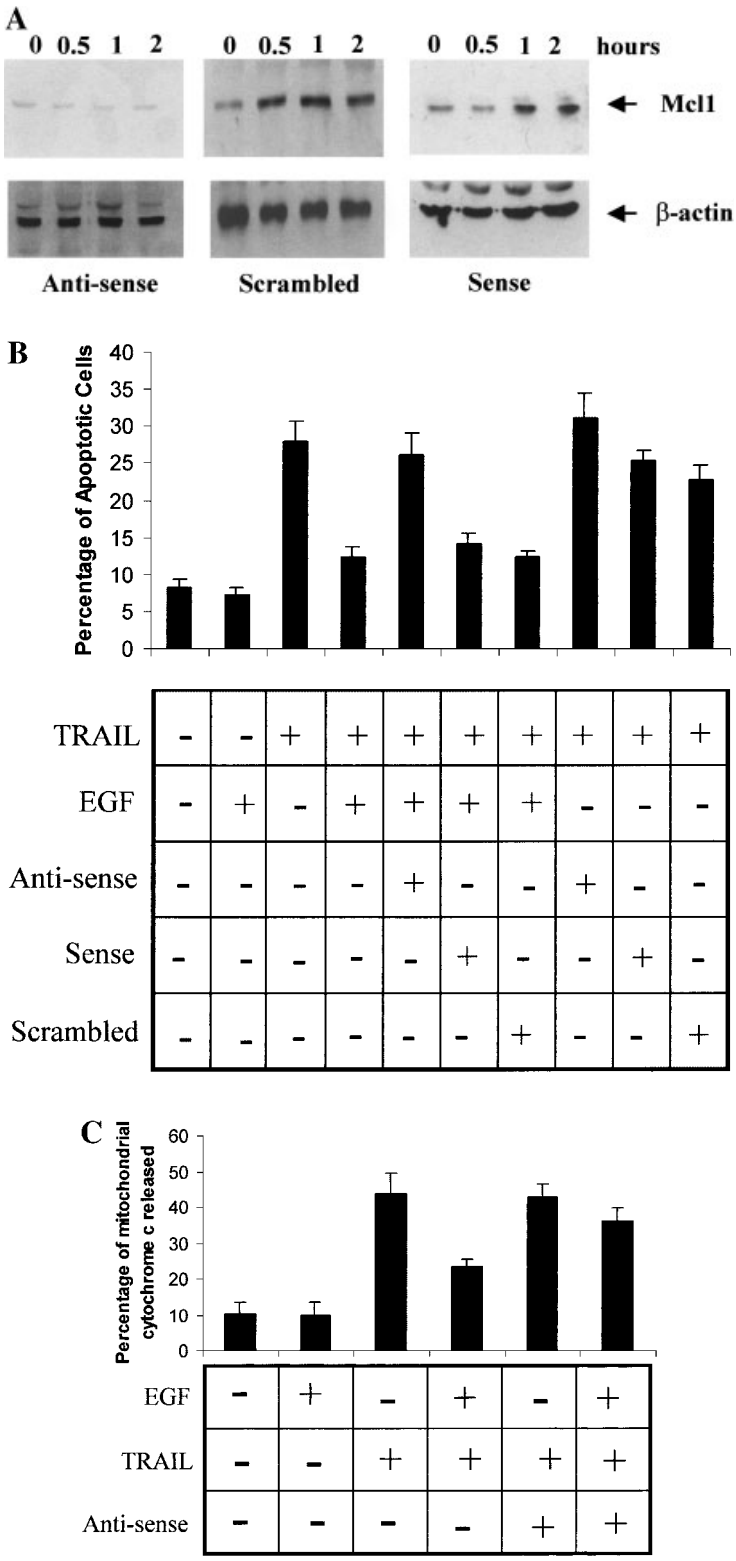
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Fig. 4.

scriptional activity) were treated with TRAIL alone or in combination with EGF and the amount of apoptosis was determined by measuring DNA condensation (Fig. 6A). HEK293 vector alone cells untreated and treated with EGF showed low levels of apoptosis (16.1 and 14.1%) that were comparable to ΔIκB cells untreated or treated with EGF (13.2 and 12.9%). ΔIκB did not alter TRAIL induced apoptosis as control and ΔIκB-expressing cells demonstrated similar amounts of apoptosis in the presence of TRAIL (36.7% for vector alone and 40.8% for ΔIκB expressing cells). As demonstrated previously, EGF blocked TRAIL-induced apoptosis (Fig. 6A). However, in the presence of ΔIκB the effects of EGF on TRAIL-induced apoptosis were essentially abrogated (41.9% apoptosis). In addition, overexpression of the p50 subunit of NFκB effectively blocked TRAIL induced apoptosis (data not shown). Thus functional NFκB is required for the ability of EGF to block TRAIL-induced apoptosis.

We previously demonstrated that EGF effectively blocks TRAIL mediated cytochrome-*c* release from the mitochondria in HEK293 cells [Gibson et al., 2002]. To determine if NFκB activation is required for the ability of EGF to block this effect of TRAIL, HEK293 vector alone, and ΔIκB cells were untreated, treated with EGF alone, treated with TRAIL alone, and in combination. The cells were then stained for cytochrome-*c* as described above. HEK293 vector alone and ΔIκB expressing cells untreated and treated with EGF alone showed punctate staining for cytochrome-*c* indicative of localization in the mitochondria (Fig. 6B). Treatment with TRAIL gives diffuse, light staining in both cell lines, which indicates the release of cytochrome-*c* from the mitochondria. However, in the vector alone cells treatment with EGF and TRAIL did not induce a redistribution of cytochrome-*c* from the mitochondria as indicated by punctate staining. In contrast, treatment of ΔIκB expressing cells with EGF and TRAIL showed light diffuse staining for cytochrome-*c*. To quantitate these results, cells were scored for cytochrome-*c* release based on the presence or absence of punctate staining; at least 200 cells were scored in three separate experiments. HEK293 vector alone cells untreated and treated with EGF showed low levels of cytochrome-*c* release from the mitochondria (9.1 and 7.7%). This was comparable to ΔIκB expressing cells untreated and treated with EGF (6.1 and 10.0%). TRAIL treatment alone showed release of cytochrome-*c* from the mitochondria in both cell lines, 43% for vector alone and 37% for ΔIκB expressing cells. However, EGF and TRAIL treatment in vector alone cells provides partial protection against cytochrome-*c* release with TRAIL treatment (21.4%) that was not found in ΔIκB expressing cells (36.8%,). This indicates that functional NFκB is required for EGF to block TRAIL induced mitochondrial cytochrome-*c* release.

EGF Activates NFκB Mediated by AKT in HEK293 and NIH3T3 Cells Expressing EGF Receptors

AKT leads to the activation of NFκB and is involved in EGF survival responses [Kane et al., 1999; Madrid et al., 2000]. We determined if EGF activation of NFκB is dependent on AKT. A gel mobility shift assay using oligonucleotides containing NFκB DNA binding consensus motifs was used to monitor EGF activation of NFκB in HEK 293 vector alone and AKT kinase inactive (AKT-KM) cells as described in Materials and Methods. Expression of AKT kinase inactive acts as a dominant negative mutant, blocking AKT phosphorylation of its anti-apoptotic substrates in several cell lines [Datta et al., 1999]. As a positive control, HEK 293 cells overexpressing p65 subunit of NFκB were used.

Fig. 4. Anti-sense oligonucleotides against Mcl-1 blocks EGF protection against TRAIL induced apoptosis and release of cytochrome-*c* from the mitochondria. HEK293 cells were transfected with anti-sense, sense, and scrambled oligonucleotides against Mcl-1 as described in Materials and Methods. **A**: The cells were then treated with EGF (1 μg/ml) for a 2 h time course with times as indicated above. Time 0 represents cells not treated with EGF. The cells were lysed and Western blotted for Mcl-1 and β-actin as a loading control. **B**: Cells were treated with EGF alone, TRAIL alone, or in combination. In addition, cells were transfected with anti-sense, sense, or scrambled oligonucleotides and treated with TRAIL or in combination with EGF as indicated. After 36 h of incubation, the cells were counted for apoptosis by acridine orange staining as described in Materials and Methods. Standard error was calculated from three independent experiments. **C**: Cells were transfected with anti-sense oligonucleotides against Mcl-1 in the presence of TRAIL or in combination with EGF. After 36 h of incubation, the cells were immunofluorescently stained with cytochrome-*c* as described in Materials and Methods. The amount of cytochrome-*c* release from the mitochondria was determined. All experiments were repeated three independent times and standard error was calculated from three independent experiments.

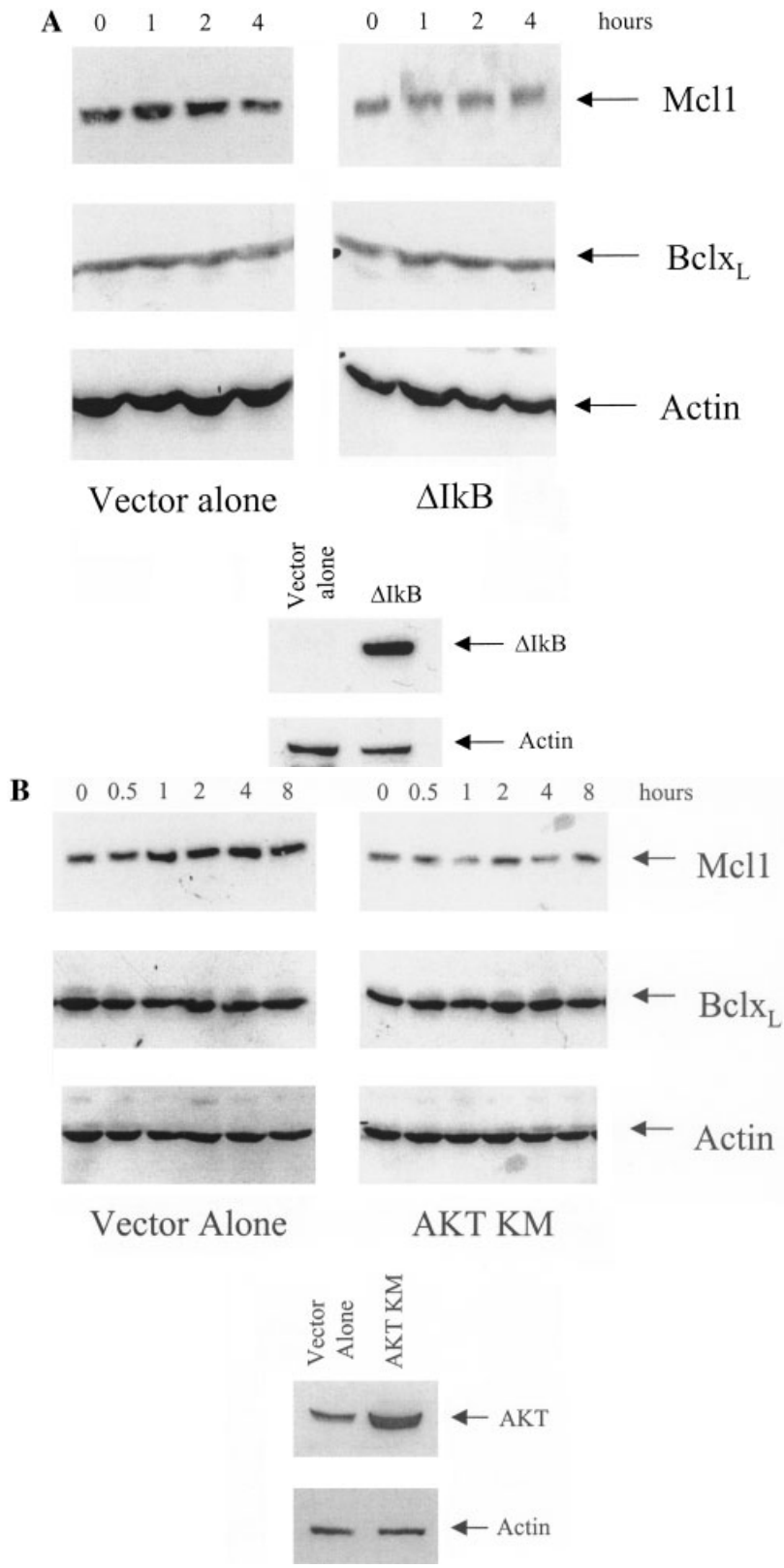


Fig. 5. Inhibition of NFκB and AKT blocks EGF mediated up-regulation of Mcl-1 (**A**) HEK 293 cells expressing vector alone or ΔIκB were treated with EGF (1 μg/ml) for a 4 h time course. The cells were lysed and Western blotted for Mcl-1, Bcl-x_L, or β-actin as described in Materials and Methods. The level of ΔIκB expression was determined by Western blotting for IκB. **B**: HEK 293 vector alone and AKT KM cells were treated with EGF as described in (A) but over an 8 h time course. The cell lysates were Western blotted for Mcl-1, Bcl-x_L, or β-actin as described in Materials and Methods. As a control, the amount of AKT-KM expression was also determined by Western blotting.

In vector alone cells, the basal levels of NFκB bound to DNA were higher than in AKT-KM cells where it is barely detectable (Fig. 7A). With EGF treatment, NFκB bound to DNA increased at 30 min peaking at 1 h in vector alone cells, however, in AKT-KM cells NFκB bound to DNA was barely detectable at 1 h (Fig. 7A). This suggests that EGF activation of NFκB requires AKT in HEK293 cells.

NIH3T3 cells that do not express ErbB receptors at significant levels (parental), express human ErbB1 alone (NE1) or express human ErbB1 and ErbB2 (NE1/2) [Olayioye et al., 2000] were used to determine the involvement of AKT in NFκB activation. In both ErbB1 and ErbB1/ErbB2 expressing NIH 3T3 cells, EGF activated AKT kinase activity (data not shown). NIH3T3 parental, ErbB1 alone expressing and ErbB1/ErbB2 expressing cells were also treated with EGF alone or in combination with wortmannin (inhibits PI3K activation thereby blocking AKT activation). The NFκB transcriptional activity was determined by a luciferase assay as described in Materials and Methods. In parental cells, there was no change in NFκB activation following EGF treatment (data not shown). NFκB transcriptional activity, however, increased 2.3-fold above basal levels in ErbB1 alone expressing cells after 1 h and peaked at 3.5-fold after 2 h. In ErbB1/ErbB2 expressing cells, NFκB transcriptional activity peaked at 2.3-fold 2 h after addition of EGF (Fig. 7B). In both ErbB1 and ErbB1/ErbB2 expressing cells, wortmannin treatment prevented NFκB activation following EGF treatment, with levels approximately equivalent to basal levels (Fig. 7B). Thus, EGF activation of NFκB following ligation of ErbB1 alone or in combination with ErbB2 depends on AKT activation similar to HEK 293 cells.

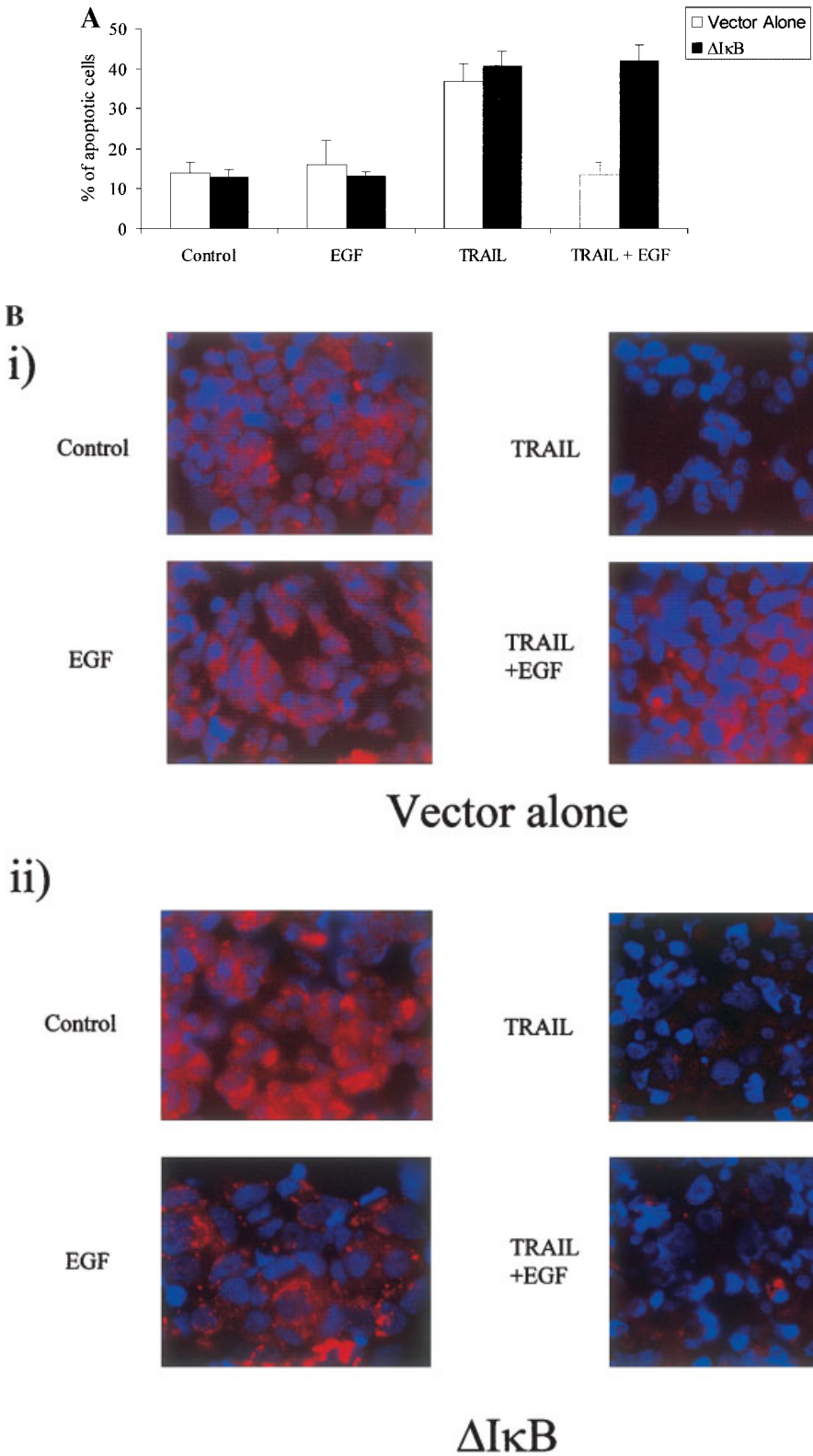
DISCUSSION

Herein we have shown that overexpression of ErbB2 or EGF binding to ErbB1 alone, or in combination with ErbB2 provides protection against TRAIL induced apoptosis. EGF through ligation of ErbB1 alone or in combination with ErbB2 leads to up-regulation of the anti-apoptotic Bcl-2 family member Mcl-1 and blocking this up-regulation with anti-sense oligonucleotides against Mcl-1 restores TRAIL induced cytochrome-*c* release and apoptosis. Blockage of NFκB and AKT activation inhibits EGF up-regulation of Mcl-1. Furthermore, EGF mediated NFκB activation prevents TRAIL mediated release of cytochrome-*c* from the mitochondria and apoptosis. These results indicate a direct signaling pathway integrating EGF signaling to inhibition of TRAIL induced apoptosis through increased Mcl-1 expression.

EGF activation of MAP kinase increases Mcl-1 levels and confers resistance to staurosporine induced apoptosis in the esophageal carcinoma (CE) cell line, CE81T/VGH [Leu et al., 2000]. It was, however, unknown whether EGF mediated AKT activation leads to increased expression of Mcl-1 and if this increase is responsible for protection against death receptor induced apoptosis. We have demonstrated that the EGF activation of ErbB1 and/or ErbB2 selectively increases the expression of Mcl-1 mediated by AKT and NFκB activation leading to blockage of TRAIL induced apoptosis. Mcl-1 also effectively blocks apoptosis by binding to pro-apoptotic protein Bax at the mitochondria preventing release of cytochrome-*c*. We have demonstrated that induction of Mcl-1 is sufficient to mediate EGF protection against TRAIL induced cytochrome-*c* release from the mitochondria. Thus, elevated Mcl-1 expression mediated by EGF protection against apoptosis.

EGF binds to many ErbB receptors but the ErbB receptors responsible for EGF protection were unknown [Pinkas-Kramarski et al., 1997]. We determined at least in NIH3T3 and HEK293 cells that ErbB1 homodimer and ErbB1/ErbB2 heterodimer is sufficient to provide EGF protection against TRAIL induced apoptosis. In addition, overexpression of ErbB2 is sufficient to protect cells from TRAIL induced apoptosis in the absence of EGF. Heregulin that binds to ErbB3 and ErbB4 [Olayioye et al., 2000] failed to protect against apoptosis in HEK293 cells (data not shown). This indicates that ErbB1 and ErbB1/2 contribute to EGF protection against TRAIL induced apoptosis mediated by Mcl-1.

Treatment of cells with platelet-derived growth factor (PDGF) or insulin-like growth factor (IGF1) leads to NFκB activation mediated by AKT and is involved in cell survival [Romashkova and Makarov, 1999a]. We have shown for the first time that EGF activates the AKT/NFκB pathway leading to protection against TRAIL induced apoptosis. Similarly, ErbB2 also activates AKT/NFκB signaling pathway leading to cell survival [Tari and Lopez-Berestein, 2000]. However, there have


A
% of apoptotic cells
50
40
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TRAIL
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Fig. 6.

been several conflicting reports regarding AKT activation of NFκB. AKT kinase activity was shown not to contribute to activation of NFκB in macrophages and ErbB receptor activation of NFκB could be independent of AKT [Biswas et al., 2000; Habib et al., 2001]. These differences could be cell type specific or stimulation dependent. Indeed, NFκB activation is regulated by other serine threonine kinases besides AKT such as MEKK1 and NIK [Lee et al., 1998]. However, our results indicate that in NIH3T3 and HEK293 cells, EGF induced NFκB activation is mediated by AKT contributing to cell survival.

NFκB activation is implicated in cell survival by up-regulation of a variety of anti-apoptotic genes including Bcl-2 family members such as Bcl-x_L, and caspases inhibitors cFLIP, and cIAP2 [Wang et al., 1998a; Krueger et al., 2001]. Expression of cFLIP inhibits caspase 8 activation but we have previously shown that EGF treatment fails to inhibit caspase 8 activation [Gibson et al., 2002]. Thus, cFLIP is an unlikely target of EGF mediated NFκB activation. IAP expression inhibits caspase activation and could be a target of EGF mediated NFκB activation but IAPs do not inhibit cytochrome-*c* release from the mitochondria [Richter and Duckett, 2000]. Bcl-x_L has been shown to block cytochrome-*c* release from the mitochondria and TRAIL induced apoptosis [Burns and El-Deiry, 2001]. However, we have demonstrated that TRAIL induces apoptosis in cell expressing Bcl-x_L and Bcl-x_L failed to be up-regulated both at the mRNA and protein levels following EGF treatment. In contrast, Mcl-1 was up-regulated by EGF mediated NFκB activation in HEK293 cells. Thus, EGF survival response in HEK 293 cells is likely mediated by NFκB induced Mcl-1 expression.

In HEK293 cells, EGF requires AKT mediated NFκB activation to increase Mcl-1 protein levels. AKT has been previously demonstrated to increase Mcl-1 protein levels but instead of involving NFκB activation, AKT activates another transcription factor CREB leading to increased Mcl-1 expression [Wang et al., 1999]. In addition, EGF leads to CREB activation [Groot et al., 2000]. It has been suggested that CREB associates with NFκB and cooperate in gene regulation [Abraham, 2000]. The role of CREB in EGF mediated Mcl-1 up-regulation in HEK 293 cells is unclear and will be investigated in the future.

TRAIL has been proposed as a molecular based treatment for cancer. Administration of TRAIL to nude mice containing human tumors ranging from breast to lung reduced the size of these tumors without toxic side effects on surrounding healthy tissues [Walczak et al., 1999]. We have previously shown that EGF protects cells against FAS and TRAIL induced apoptosis [Gibson et al., 1999, 2002]. In addition, Herceptin^{TM} that binds and inactivates ErbB2 signaling increased sensitivity of cells to TRAIL induced apoptosis [Cuello et al., 2001]. This protection seems to involve blockage of cytochrome-*c* release from the mitochondria. TRAIL induced apoptosis also requires the pro-apoptotic member Bax to release mitochondrial proteins such as cytochrome-*c* and Smac/Diablo [Deng et al., 2002; LeBlanc et al., 2002]. Our results seem to agree with these findings since Mcl-1 expression blocks TRAIL induced mitochondrial cytochrome-*c* release and apoptosis and Mcl-1 is increased in breast tumors with overexpression of ErbB2. Furthermore, Mcl-1 can block Bax induced apoptosis similar to other Bcl-2 family members [Wang et al., 1998b]. Overexpression of Bcl-2 can also block TRAIL induced apoptosis [Fulda et al., 2002]. Besides TRAIL induced apoptosis, genotoxins such as etoposide induce TRAIL death receptor expression and blockage of TRAIL death receptor activation inhibits etoposide induced apoptosis [Gibson et al., 2000]. Taken together, up-regulation of Mcl-1 in cancer cells could reduce TRAIL sensitivity as well as sensitivity of other chemotherapeutic drugs requiring activation of death receptor apoptotic pathways.

Fig. 6. Blockage of NFκB activation relieves EGF protection against TRAIL induced apoptosis. HEK293 vector or ΔIκB cells were treated with EGF (1 μg/ml) or TRAIL (1 μg/ml) alone and in combination. Control cells were left untreated. **A**: After 24 h, the amount of apoptosis was quantified by acridine orange staining. Cells were scored as apoptotic when the staining showed condensed DNA as compared to evenly stained nuclei. Cells were counted on an Olympus CK40 fluorescent microscope. At least 200 cells were counted in three separate experiments; error bars represent the standard error. **B**: HEK293 vector alone cells (i) or ΔIκB cells (ii) were grown on coverslips and then treated with EGF (1 μg/ml) or TRAIL (1 μg/ml) alone and in combination. Control cells were left untreated. After 24 h, slips were removed and fixed in 3.7% formaldehyde and stained by immunohistochemistry for cytochrome-*c* (red). The nucleus was counterstained with Hoechst (blue) and the cells were visualized on a Ziess Axiophot and images captured with a digital camera.

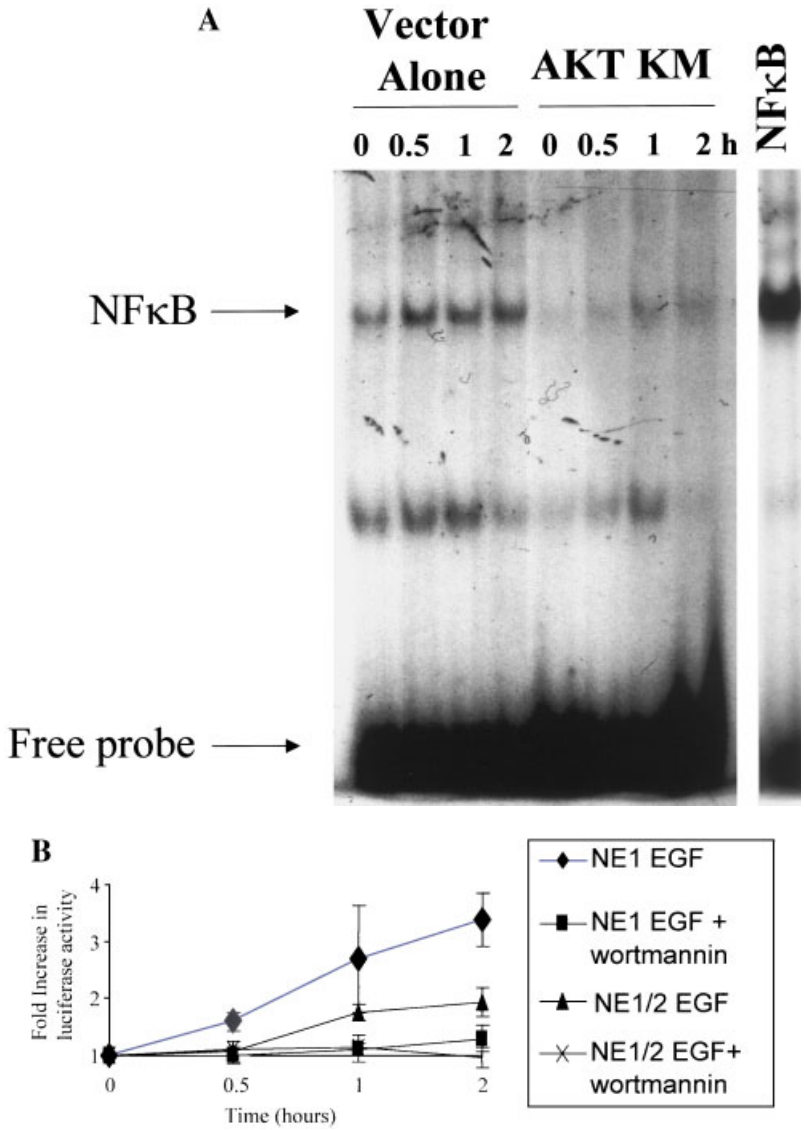

Fig. 7. EGF activation of NFκB is mediated by AKT in HEK293 and NIH3T3 cells. **A**: HEK293 vector or AKT KM cells were treated with EGF (1 μg/ml) for the times indicated and the cells were lysed in NP40 lysis buffer, and the nuclear fraction isolated by centrifugation. Double stranded oligonucleotides containing the NFκB DNA binding site (Santa Cruz Biotechnology, Santa Cruz, CA) were radioactively labeled with γP^{32}ATP, and then added to the nuclear extracts. Oligonucleotide-nuclear extract mix was run out on a 5% native polyacrylamide gel, the gel dried, and then exposed overnight to X-ray film. This represents three independent experiments. **B**: NIH3T3 parental cells do not express ErbB receptors, NE1 cells express ErbB1 receptors exclusively (NE1), NE1/2 cells co-express ErbB1 and ErbB2 (NE1/2). NIH3T3 cells were serum starved overnight and then treated with EGF (1 μg/ml) and lysed in kinase lysis buffer cells were transiently transfected with 1 μg of the NFκB luciferase reporter gene and 0.2 μg of the β-gal reporter gene using the GenePorter transfection reagent as per manufactures instructions. Transfection efficiency was determined by an enzymatic assay for β-gal (Promega, Madison, WI) read on an ELISA reader. Eighteen hours post-transfection cells were treated with EGF alone or in combination with (100 nM) wortmannin and lysed in 5× lysis buffer for luciferase (Promega). Luciferase activity was measured after the addition of luciferin reagent (Promega) in a microtiter plate luminometer and represented as fold increase in luciferase activity compared to untreated cells. Error bars represent standard error of the mean.

Anti-sense oligonucleotides against specific proteins have been proposed as another treatment for cancer [Huang, 2000]. In particular, anti-sense oligonucleotides against Bcl-2 have been successfully used to treat a variety of leukemias with high Bcl-2 expression levels [Huang, 2000]. It is most effective in combination with traditional chemotherapy. Anti-sense oligonucleotides against Mcl-1 inhibit the EGF protective effects against TRAIL induced

apoptosis. In human myeloma cells, anti-sense against Mcl-1 induces apoptosis [Derenne et al., 2002]. This indicates that use of anti-sense oligonucleotides against Mcl-1 in combination with TRAIL could be an effective treatment for cancers that are resistant to TRAIL treatment alone such as breast cancers overexpressing ErbB receptors. This will be the focus of future investigations.

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