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Regulation of ER α -mediated transcription of Bcl-2 by PI3K-AKT crosstalk: Implications for breast cancer cell survival

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Abstract

Both estrogen, through the estrogen receptor (ER), and growth factors, through the phosphatidylinositol-3-kinase (PI3K)-AKT pathway, have been shown to independently promote cell survival. Here, we investigated the role of ER/PI3K-AKT crosstalk in the regulation of cell survival in MCF-7 breast carcinoma cells. The ER inhibitor ICI 182,780 was used to determine the requirement of the ER for estrogen in the suppression of tumor necrosis factor- α (TNF α) induced apoptosis. Gene reporter assays and Western blot analyses were used to determine the involvement of the pro-survival factor Bcl-2 and the coactivator GRIP1 in this survival crosstalk. We demonstrated that an intact ER signaling pathway was required for estrogen to suppress apoptosis induced by TNF α . Our gene reporter assays revealed that ER α , not ER β , was targeted by AKT, resulting in transcriptional potentiation of the full-length Bcl-2 promoter, ultimately leading to increased Bcl-2 protein levels. AKT targeted both activation function (AF) domains of the ER α for maximal induction of Bcl-2 reporter activity, although the AF-II domain was predominately targeted. In addition, AKT also caused an upregulation of GRIP1 protein levels. Finally, AKT and GRIP1 cooperated to increase Bcl-2 protein expression to a greater level than either factor alone. Collectively, our study suggests a role for ER/PI3K-AKT crosstalk in cell survival and documents the ability of AKT to regulate Bcl-2 expression via differential activation of ER α and ER β as well as regulation of GRIP1.

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Keywords

estrogen receptor; breast cancer; AKT; cell signaling; cell survival

Introduction

Homeostasis in normal breast tissue is maintained by a balance between cell survival and cell death (1). Hence, dysregulation of this homeostasis in favor of cell survival can lead to cell proliferation and cancer (2). Estrogen (E_2) is an endogenous factor that plays a critical role in normal mammary functions (3). However, E_2 has been implicated in breast cancer due to its pro-survival effects (4,5). The phorbol ester insulin-like growth factor 1 (IGF-1), is also involved in cell survival and its effects are largely mediated by the phosphatidylinositol 3 kinase (PI3K)-AKT signaling cascade (6). While there are many downstream targets of PI3K, the serine/threonine kinase AKT is the primary mediator of PI3K survival signaling due to AKT's ability to regulate cellular components that affect cell survival decisions, such as Bad and Forkhead transcription factors (7).

The actions of E_2 are mediated by the estrogen receptor (ER). The two isoforms of the ER, ER α and ER β , belong to the steroid/thyroid hormone superfamily of nuclear receptors that function as ligand-activated transcription factors (8). Transcriptional activities of the receptors are mediated by two distinct activation function (AF) domains: the AF-I domain in the amino terminus exhibits constitutive ligand-independent activity, and the AF-II domain in the carboxy-terminus requires ligand binding for activity (9). To achieve full transcription potential, the ER must also recruit histone modifying coactivators to overcome the steric hindrances imposed on tightly packed chromatin (10,11). Glucocorticoid receptor-interacting protein 1 (GRIP1), a member of the p160 family of coactivators, has been shown to interact with both AF domains of the ER (12-14), increasing the transcriptional activity of the receptor (15). Several coactivators have been implicated in breast cancer etiology (16-18), and researchers have recently begun to study peptide growth factor regulation of coactivator function to better understand cancer development (19-21).

Both E_2 (22-24) and IGF-1 (25-27) enhance cancer cell survival in part through their abilities to upregulate Bcl-2 expression. A cAMP response element (CRE) within the Bcl-2 promoter is targeted by IGF-1 signaling via AKT phosphorylation of the cAMP response element binding protein (CREB) (26,27). Likewise, the ER has been shown to play an integral role in E_2 regulation of Bcl-2 expression (28-30). However, the exact role of the ER at the Bcl-2 promoter remains unclear. A survival crosstalk between the ER- E_2 and IGF1-PI3K-AKT signaling pathways (31-33) may protect cancer cells from apoptosis induced by the chemotherapeutic drug tamoxifen (34) and the microbial product wortmannin (35). In these cases, peptide growth factor activation of the PI3K-AKT signaling cascades potentiated ER α transcriptional activity (31,33,34). Interestingly, the CRE site within the Bcl-2 promoter is flanked by two EREs, making the Bcl-2 promoter a possible point of convergence for this ER- E_2 /PI3K-AKT survival crosstalk.

The endogenous cytokine tumor necrosis factor α (TNF α) has pleiotropic biological functions (36) and is produced by a variety of cell types in both normal (37) and breast cancer tissue (38). TNF α induces apoptosis in normal breast tissue during involution (39) and in breast cancer cells (40). TNF α -induced apoptosis is inhibited by Bcl-2 (41). Hence, factors that control Bcl-2 expression may increase cell survival and contribute to breast cancer growth, even in the presence of TNF α (36,42).

Given the roles of estrogen and peptide growth factors in breast cancer biology, we hypothesize that PI3K-AKT-ER α -E₂ crosstalk enhances cancer cell survival. We evaluated the ability of ER/PI3K-AKT crosstalk to affect MCF-7 cells at both the biological and molecular levels. Our results indicate that activation of the PI3K-AKT pathway contributes to breast cancer cell survival through transcriptional activation of the Bcl-2 gene product in an ER α -dependent manner, which may increase breast cancer cell survival in the presence of TNF α (43). Given the potential of TNF α use in breast cancer therapy (36,42), a mechanistic study of this PI3K-AKT-ER α -E₂ survival crosstalk was investigated.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), phenol-red free DMEM, fetal bovine serum (FBS), BME amino acids, MEM amino acids, L-glutamine, penicillin, streptomycin, and sodium pyruvate were obtained from Gibco-BRL (Gaithersburg, MD). Porcine insulin was purchased from Sigma (St. Louis, MO), and charcoal stripped (CS) FBS was obtained from HyClone (Logan, UT). Lipofectamine and Effectene were purchased from Gibco-BRL (Grand Island, NY) and QiaGen (Valencia, CA), respectively. ICI 182,780 was obtained from Tocris (Ellisville, MO). TNF α was obtained from R&D systems (Minneapolis, MN). 17 β -estradiol (E₂) and all protease inhibitor cocktails were purchased from Sigma; NuPAGE 4-8% Bis-Tris gel was obtained from Invitrogen (Carlsbad, CA); Bio-Rad protein assay reagent was purchased from Bio-Rad (Hercules, CA). The primary antibodies, mouse anti-human Bcl-2, rabbit anti-GRIP1, and rabbit anti-actin, were obtained from BD PharMigen (San Diego, CA), Upstate Biotechnology (Lake Placid, NY) and Sigma, respectively. The secondary antibodies, horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit, were purchased from Transduction Laboratories (Lexington, KY) and Cell Signaling (Beverly, MA), respectively. ECL chemiluminescence system was obtained from Amersham (Buckinghamshire, England), and Biomax film was purchased from Kodak (Rochester, NY). The inverted fluorescence microscope was purchased from Leica (Wetzlar, Germany), and the Monolight 2010 luminometer was obtained from Analytical Luminescence Laboratory (Ann Arbor, MI).

Plasmids

pGL3-Con-Luc, pEGFP-N1, and dominant negative AKT (AKT-DN) were obtained from Promega (Madison, WI), Clontech (Palo Alto, CA), and Upstate Biotechnology, respectively. The following expression vectors have been previously described: constitutive active AKT (AKT-CA) (44), pcDNA3.1-ER α and β (45). pRST7-ER α and pRST7-ER β constructs (AF-I with alanine substituting for amino acids at positions 436, 440, and 443; AF-II containing amino acids 90-477) were generously provided by Dr Donald McDonnell (46). Bcl-2-Luciferase reporter construct (-3934 to -1287 basepairs upstream from the transcription start site) was a gift of Dr Martin P. Smith and Dr Linda Boxer (47).

Cell culture

Estrogen receptor positive MCF-7 human breast carcinoma (48) and estrogen receptor negative human embryonic kidney (HEK) 293 cells (49) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, BME amino acids, MEM amino acids, L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, sodium pyruvate, and 1×10^{-10} M porcine insulin under myco-plasma-free conditions at 37°C in humidified 5% CO₂ and 95% air. For described studies, MCF-7 cells were grown for 48 h in phenol red-free DMEM supplemented with 5%-charcoal stripped (CS) FBS, and supplements as above, but without insulin (5% CS-DMEM), as previously described (23).

Transient transfection and luciferase assay

MCF-7 and HEK 293 cells were transfected as previously described (23). Briefly, cells were placed in 5% CS-DMEM for 48 h prior to plating onto 24-well plates at 1×10^4 cells/well and allowed to attach overnight. The next day, the cells were transfected for 5 h in the serum/supplement-free DMEM using either Lipofectamine or Effectene according to the manufacturers' protocols. For reporter-based luciferase assay, MCF-7 and HEK 293 cell were transfected with the reporter (Bcl-2-Luc), along with vector control (Vec), AKT-CA, or AKT-DN, with 293 cells additionally transfected with various ER α or ER β constructs. After 5 h, the transfection medium was replaced with 5% CS-DMEM, and treated with chemicals (vehicle control or 1 nM E₂, with or without 100 nM ICI 182,780). After 24 h, the treatment-containing medium was removed and 1X lysis buffer (100 μ l) was added per well and gently shaken for 30 min at room temperature. The cell debris was then pelleted by centrifugation at $15,000 \times g$ for 5 min. Luciferase activity for 30 μ l of cell extract was determined using luciferase assay substrate in a Monolight 2010 luminometer.

Viability assay

Viability assay using crystal violet (CV) was performed as previously described (44). Briefly, MCF-7 cells (1×10^4 /well) in 24-well plates with 5% CS-DMEM were treated with 10 ng/ml TNF- α , with or without 1 nM E₂, with or without 100 nM ICI 182,780 for 24 h, stained using 0.5% crystal violet (200 μ l) for 15 min, washed twice with phosphate buffered saline (PBS), lysed in 1% SDS solution (600 μ l), transferred to a 96 well plate, and absorbency (ABS) at 550 nm was measured. ABS of treated samples was normalized to untreated control (100%). For reporter gene viability assay, cells were transfected with pGL3-Luc reporter and empty vector (Vec) or constitutive active AKT (AKT-CA), treated for 48 h with 10 ng/ml TNF- α , with or without 1 nM E₂, with or without 100 nM ICI 182,780 and harvested for luciferase assay with data represented as percent viability normalized to untreated control ($100\% \pm \text{SEM}$). Apoptosis analysis with fluorescence microscopy was performed as previously described (50) with the use of green fluorescence protein (GFP) instead of LacZ as a marker of transfection. Cells were transfected with pEGFP (100 ng) and 500 ng empty vector (Vec) or constitutive active AKT (AKT-CA) using Effectene, followed by treatment with 10 ng/ml TNF- α , with or without 1 nM E₂, with or without 100 nM ICI 182,780 for 24 h, after which cells were fixed in 4% paraformaldehyde and washed twice with PBS. Apoptotic (rounded) and normal GFP expressing cells were visualized using a Leica inverted fluorescence microscope.

Immunoblot analysis

MCF-7 and HEK 293 cells were seeded at 5×10^6 cells/100 mm² plate. MCF-7 cells were transfected with a total of 5 μ g DNA of empty vector (Vec), constitutive active AKT (AKT-CA), or pSG5-GRIP1-HA (GRIP1), or a combination of AKT-CA and pSG5-GRIP1-HA. The 293 cells were additionally transfected with ER α . Cell were transfected using Lipofectamine for 5 h, then treated with vehicle control or 1 nM E₂ with or without 100 nM ICI 182,780 for 24 h. Cells were washed twice in 4°C PBS, pH 7.2, harvested in sonicating buffer (62.5 mM Tris-HCl, pH 6.8, 4% w/v SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ l/ml protease inhibitor cocktail), and sonicated for 30 sec on ice. Following centrifugation at $1,000 \times g$ for 20 min and determination of protein concentration using Bio-Rad protein assay, 50 μ g of protein was resuspended in sample loading buffer (62.5 M Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 5% β -mercaptoethanol, 0.01% bromophenol blue), boiled for 3 min and electrophoresed onto NuPAGE 4-8% Bis-Tris gel. The proteins were then transferred electrophoretically to a nitrocellulose membrane, and the membrane was blocked with PBS/0.05% Tween/5% low-fat milk solution at 4°C overnight with gentle shaking. The membrane was subsequently incubated with 1 μ g/ml mouse anti-human Bcl-2 or 4 μ g/ml rabbit anti-GRIP1 antibody for

2 h at room temperature with gentle shaking. The blot was washed in PBS/0.05% Tween solution and incubated with 1:5,000 dilution of HRP-conjugated goat anti-mouse or 1:2,000 dilution of HRP-conjugated anti-rabbit antibody for 30 min at room temperature with gentle shaking. Following four washes with PBS/0.05% Tween solution, immuno-reactive proteins were detected using ECL chemiluminescence system and recorded by fluorography on Kodax Biomax film according to the manufacture's instructions. The membrane was then stripped in stripping solution (2% SDS, 62.5 mM Tris-HCl, pH 6.7, 7 μ l/ml β -mercaptoethanol) for 12 min at 60°C, and the same protocol as above was used to re-probe the membrane with 1:200 dilution of rabbit anti-actin antibody and subsequently with 1:3,000 dilution of anti-rabbit antibody. Fluorograms were quantitated by image densitometry using Quality One program for data acquisition and analysis (Bio-Rad, Hercules, CA).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and post-hoc Tukey's multiple comparisons with Origin 7.0 software. Statistically significant changes were determined at the $p < 0.05$ level as indicated for each figure.

Results

The ER is required for cell survival signaling through AKT

Both estrogen, acting through the ER (51) and peptide growth factors, working through PI3K-AKT (52), can suppress apoptosis induced by various agents. To determine whether the previously described crosstalk between these two signaling pathways (31-35) can protect MCF-7 breast carcinoma cells from apoptosis induced by the endogenous cytokine TNF α , cell viability assays were performed. TNF α (10 ng/ml) caused a significant decrease in MCF-7 cell survival compared to untreated control cells (Fig. 1A). E₂ (1 nM) functioned as a potent cell survival signal by elevating cell survival to the levels of untreated control cells (95.2 \pm 4.3% viability with E₂) (Fig. 1A). Not surprisingly, ablation of ER signaling with the pharmacologic inhibitor ICI 182,780 (1 μ M) abrogated the anti-apoptotic activity of E₂.

Since AKT is involved in cell survival, the ability of this kinase to mediate cell survival in the presence of TNF α was investigated. A viability assay was performed in MCF-7 cells transfected with either empty vector (Vec) or constitutive active AKT (AKT-CA) (Fig. 1B). In the presence of TNF α (10 ng/ml), AKT-CA elevated cell survival from 55.9 \pm 5.4% to 95.7 \pm 3.1%. However, AKT protection was reduced to 47.2 \pm 6.3% in the presence of ICI 182,780 (100 nM), again suggesting that the ER is required for AKT-mediated cell survival. Cell death was confirmed morphologically with fluorescence microscopy of GFP-transfected cells that were subjected to the same treatments as above (Fig. 1C). These results suggest that the ER is required for reversal of TNF α -induced apoptosis.

The ER is required for potentiation of Bcl-2 expression by AKT

The transcriptional activity of the ER is important for cell survival (53), since the receptor upregulates the expression of gene products required for long-term cell survival, such as Bcl-2. Since E₂ has been shown to increase Bcl-2 expression (mRNA) via a transcriptional mechanism (24), we investigated the roles of AKT and ER in the regulation of Bcl-2 expression. In contrast to previous results, our initial reporter-based luciferase assays of the full-length Bcl-2 promoter reveal that E₂ alone did not induce significant Bcl-2 promoter activation (Fig. 2A). Differences in promoter regions may contribute to these variations, as our Bcl-2 reporter construct i) lacks the estrogen responsive elements located within the coding region of the gene that is required for E₂ induction of Bcl-2 promoter activity (29), and ii) contains π 1 binding sites that have been shown to negatively regulate Bcl-2

expression in reporter gene assays (54). More importantly, our results indicate that in the presence of 1 nM E₂, AKT-CA potentiated Bcl-2 expression by 54-fold over Vec (Fig. 2A). Additionally, ICI 182,780 abolished this Bcl-2 upregulation, demonstrating that the ER was required for transcriptional activation of the Bcl-2 promoter by AKT.

Immunoblot analysis was used to determine whether upregulation of the Bcl-2 promoter translates into greater protein production. Previous studies exploring AKT regulation of Bcl-2 focused on the ability of this kinase to activate the Bcl-2 promoter region with reporter assays (26,27), and Western blot analyses did not account for loading controls (34). Compared to untreated vector control, E₂ (1 nM) expectedly enhanced Bcl-2 protein levels, and AKT-CA increased Bcl-2 expression even further (Fig. 2B and C). AKT-CA enhanced ligand-independent expression of Bcl-2, perhaps not surprisingly since peptide growth factor (PGF) signaling itself can increase Bcl-2 expression (26,27). More importantly, both E₂ and AKT-CA upregulation of Bcl-2 protein was blocked by ICI 182,780, again demonstrating a requirement for the ER. These results reveal that transcriptional upregulation of the Bcl-2 pro-survival gene may be one of the mechanisms by which the ER and AKT cooperate to enhance cell survival.

AKT potentiates Bcl-2 expression through ER α

Even though ER α and ER β are co-expressed in mammary tissues and breast carcinomas (55), the two receptors are differentially influenced by growth factor signaling (56). To determine the specificity of PI3K-AKT crosstalk with each ER (α or β), human embryonic kidney (HEK) 293 cells lacking both ER α and ER β were used (49). Since our earlier results suggest a requirement for the ER in Bcl-2 promoter activation, similar experiments were used to determine the involvement of ER α and ER β . In the presence of E₂ (1 nM), AKT potentiation of Bcl-2 promoter activation was shown to require ER α but not ER β (Fig. 3A). Although ER α potentiated Bcl-2 reporter activity by 5-fold over Vec alone, this increase was much less than the 54-fold potentiation observed in Fig. 2A, suggesting that both ERs may be required for the robust potentiation seen in the MCF-7 cells that contain both ER isoforms.

ER α contains two activation function domains (AF-I and AF-II) that recruit coactivator proteins and are responsible for the transcriptional activities of the receptor. Here, expression vectors for ER α that have been mutated to functionally lack either AF domain were used to examine the specificity of AKT potentiation of ER α activity. AKT-CA potentiated Bcl-2 expression through the full-length ER α , the ER α -AF-I and AF-II domains, indicating that AKT-CA may target both activation domains of ER α (Fig. 3B). AKT-CA potentiated Bcl-2 expression to the highest levels through the full-length ER α . Since the AF-I and AF-II domains synergize for maximal ER transcriptional activity (15), the ability of AKT to target both AF domains of the ER α is likely necessary for maximal potentiation of Bcl-2 expression.

To confirm the crosstalk between ER α and AKT leads to an increase in Bcl-2 protein levels, Western blot analyses were performed in HEK 293 cells transfected with AKT-CA and ER α (Fig. 3C and D). E₂ (1 nM) induction of Bcl-2 protein expression was enhanced with ER α , although the increase was not significant compared to vector control cells. In these cells containing only ER α , AKT-CA did potentiate Bcl-2 protein expression in the absence of E₂ (Fig. 3D), as seen in Fig. 2C. However, the presence of E₂ had little effect on Bcl-2 expression. Because these cells do not contain ER β , Bcl-2 potentiation may not be maximized. Collectively, our results suggest that AKT regulation of cell survival decisions require ER α , although the ER β may also be important.

AKT increases GRIP1 protein expression

Coactivators are integral components in ER-mediated transcriptional signaling (10). Here, we investigated AKT regulation of GRIP-1 protein levels using immunoblot analyses of MCF-7 cells (Fig. 4A and B). E₂ alone was not able to enhance GRIP-1 protein expression in vector control cells. However, AKT-CA did enhance GRIP-1 protein expression by 1.8-fold and by 2.14-fold in the presence of 1 nM E₂. These results suggest that AKT plays a role in expression of the coactivator GRIP-1.

AKT and GRIP-1 cooperatively enhance Bcl-2 expression

Since AKT was able to enhance GRIP-1 protein expression, we investigated the possibility that GRIP-1 may potentiate Bcl-2 expression in the presence of E₂. As previously shown, AKT-CA alone was able to potentiate Bcl-2 expression through ER α (Fig. 3B and C). Overexpression of GRIP-1 alone was not able to achieve this potentiation (Fig. 5A). However, AKT-CA and GRIP-1 potentiated Bcl-2 expression to higher levels than either factor alone. This potentiation is seen with all ER α constructs, although the effects were lowest with the AF-I mutant (Fig. 5B) and highest with the full-length (Fig. 5A). Collectively, these data suggest that AKT-CA and GRIP-1 together may preferentially target the AF-II domain to potentiate Bcl-2 expression.

To determine whether AKT-CA and GRIP1 potentiate Bcl-2 protein expression in the presence of E₂, we used immunoblot analyses. Both AKT-CA and GRIP-1 were able to potentiate Bcl-2 protein expression to similar levels, but not significantly different from vector (data not shown). However, in the presence of E₂, AKT-CA and GRIP-1 together were able to significantly enhance Bcl-2 protein expression to a greater level than either factor alone (Fig. 6A and B), suggesting that these two factors may cooperate to transcriptionally activate the Bcl-2 promoter region.

Discussion

Crosstalk between the ER-E₂ and the IGF1-PI3K-AKT signaling pathways has been shown to promote cancer cell survival. While growth factor signaling pathways have been shown to activate the ER, the precise role of the receptor in this survival crosstalk remains unclear. Previously, Campbell *et al* demonstrated the ability of AKT to rescue cells from apoptosis induced by the chemotherapeutic drug tamoxifen (34). However, since tamoxifen is an ER antagonist (57), the contribution of ER to AKT-induced cell survival could not be completely assessed. Indeed, Campbell's studies suggest that AKT survival mechanisms may be ER-independent. Recently, Boland's group demonstrated that E₂ may protect murine skeletal muscle cells from H₂O₂-induced apoptosis through ER α and ER β , possibly involving PI3K/AKT signaling (58). Our studies with a physiological inducer of apoptosis, TNF α (43), reveal that the ER is required for AKT-mediated cell survival in breast carcinoma cells (Fig. 1B), suggesting that the PI3K-AKT and ER-E₂ signaling pathways converge to regulate cell survival decisions.

The ER's ability to regulate transcription of target genes, such as Bcl-2, has been linked to its ability to protect breast cancer cells from TNF α -induced apoptosis (24,53). Here, we show that ER α , both AF-I and AF-II domains, is targeted by AKT to bring about potentiation of Bcl-2 expression at both the transcriptional and translational levels (Figs. 2 and 3). These results re-enforce the role of the ER α as a mediator of cell survival *in vitro* (59) and *in vivo* (60). Even though ER β was not able to activate the Bcl-2 promoter in response to AKT, this receptor isoform may play a secondary supportive role, since AKT was able to potentiate Bcl-2 promoter activity much more potently in cells containing both receptor isoforms (MCF-7) than in cells with only ER α (HEK 293). Recently, we showed

that ER β is targeted by AKT signaling (21), as demonstrated by the ability of AKT to potentiate ER β transcriptional activity at a consensus ERE promoter. Hence, AKT regulation of ER β function in cell survival decisions may be more important in cells or tissues where ER β expression predominates, such as in the prostate (55).

The exact role of the ER at the Bcl-2 promoter remains to be determined. Since the ER is involved in both long-term transcriptional regulation of genes (genomic effects) (61,62) and in immediate cytoplasmic signaling events (non-genomic effects) (63-67), these two functions of the ER may converge at the Bcl-2 promoter. The ER-E₂ complex upregulates Bcl-2 expression either directly by acting on EREs located within the coding region of the gene (29) or indirectly through interaction with the Sp1 protein (28). In addition, the ER may also complex with components of cytoplasmic signaling pathways, such as PI3K (68).

Previously, we showed that the overexpression of coactivators may provide a survival advantage to breast carcinoma cells in the presence of TNF α (69). Other researchers have found that the coactivator PELP1 may affect breast cancer cell sensitivity to apoptosis induced by TNF α (70). Here, we show for the first time the ability of AKT to upregulate GRIP-1 protein expression (Fig. 4) and the cooperation between AKT and GRIP-1 to enhance Bcl-2 transcriptional and protein expression to levels higher than either factor alone (Fig. 5). These results suggest that AKT potentiation of the Bcl-2 promoter region may result from AKT's ability to regulate both ER α and factors that interact with the receptor, such as GRIP-1. Once expressed, GRIP-1 may cooperate with AKT to enhance ER α activity in order to potentiate Bcl-2 expression. Other investigators have demonstrated that the coactivator amplified in breast cancer 1 (AIB1/SRC-3) is required for Bcl-2 expression. However, these investigators suggested that the regulation of Bcl-2 protein expression occurs independently of the ER (71). It is possible that GRIP-1 and AIB1 may affect the ER differentially at the Bcl-2 promoter.

Both the ER-E₂ and PI3K-AKT signaling pathways have been shown to independently protect breast cancer cells from apoptosis induced by TNF α (44,50,72). In this study, we provide evidence that a survival crosstalk between the ER-E₂ and PI3K-AKT pathways protects breast carcinoma cells from TNF α -induced apoptosis. Given the importance of TNF α in cell survival regulation of both normal and malignant breast tissue (39,40), the ability of a survival crosstalk to enhance cell survival may lead to cancer development. A growing area of research explores the potential of TNF α and its family members in cancer therapy (73-77). Hence, molecular studies of survival crosstalk that compromise the apoptotic effects of TNF α may prove therapeutically useful (36).

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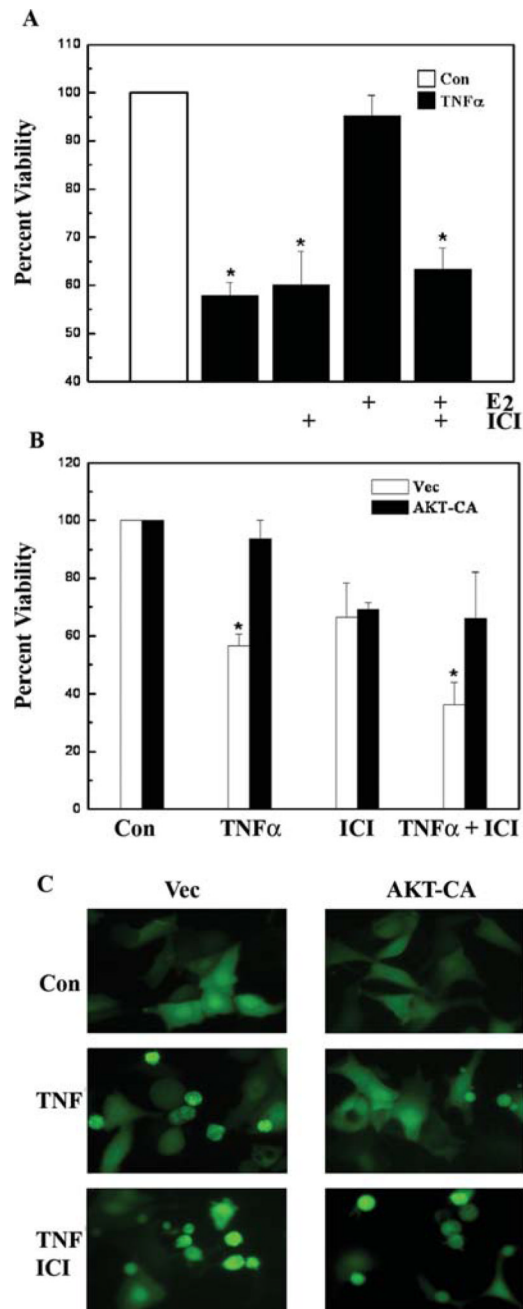
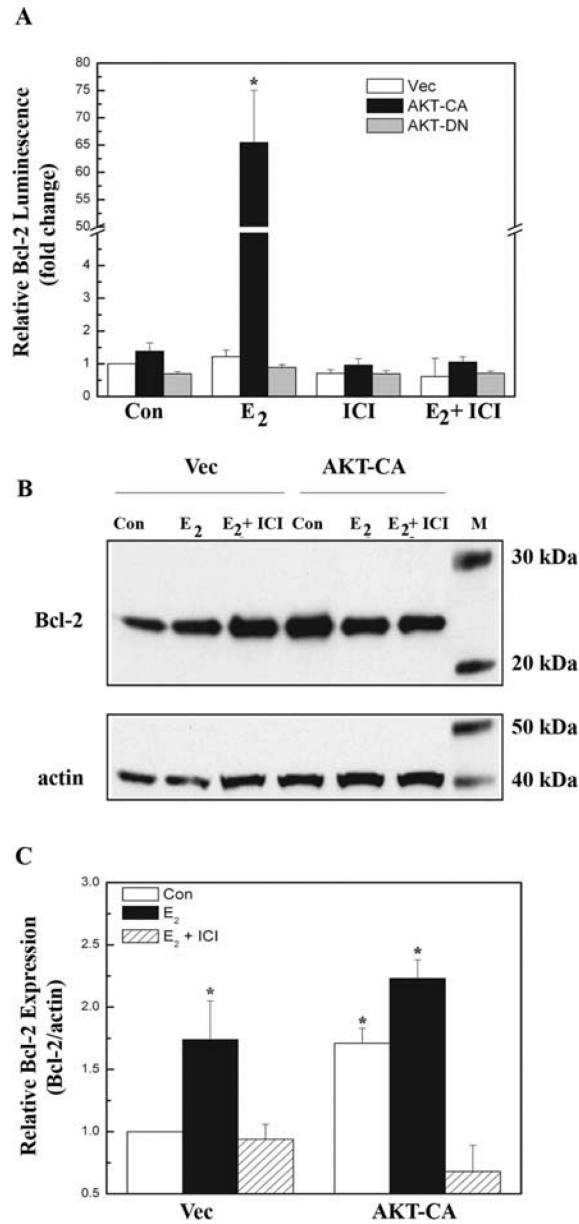


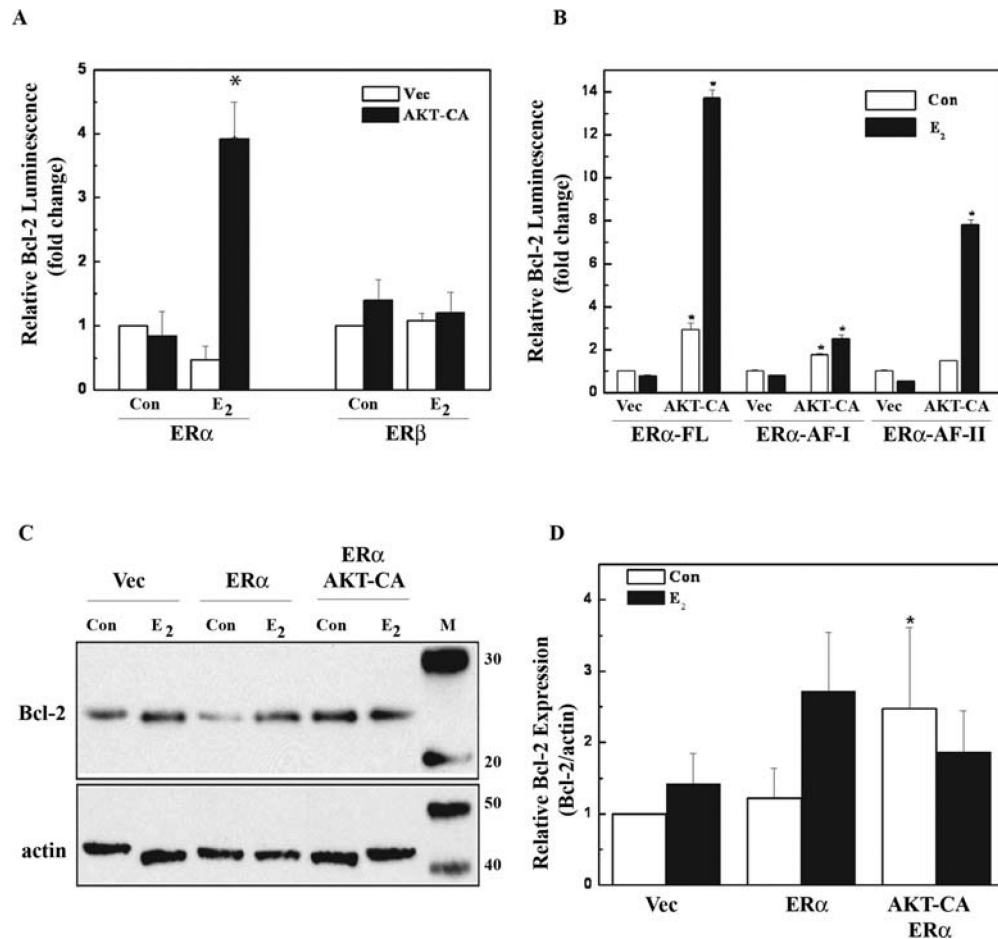
Figure 1. The ER is required for cell survival mediated by signaling through AKT. (A) MCF-7 cells were treated with vehicle control (Con) or E₂ (10 nM) in the presence or absence of ICI 182,780 (100 nM) for 24 h and then exposed to TNF α (10 ng/ml). Cells were harvested for CV viability assay 24 h later. Asterisk denotes statistical significance from control (p<0.05). (B) MCF-7 cells were transfected with 2 μ g of empty vector (Vec) or constitutive active AKT (AKT-CA) along with pGL3-Luc (200 ng). Cells were treated with vehicle (Con) or ICI 182,780 (100 nM) followed by addition of TNF α (10 ng/ml) for 48 h and harvested for viability assay. Asterisk denotes statistical significance from vector control (p<0.05). All data are the means and standard errors of double treatments from a single experiment, and are representative of at least two independent experiments. (C) MCF-7 cells were transfected

with 500 ng of empty vector (Vec) or constitutive active AKT (AKT-CA) along with a pEGFP expression vector (100 ng). Cells were treated with vehicle control (Con) or ICI 182,780 (100 nM), followed by the addition of vehicle or TNF- α (10 ng/ml) and analyzed for cell death by fluorescence microscopy 24 h later.

**Figure 2.**

The ER is required for potentiation of Bcl-2 expression by AKT. (A) MCF-7 cells were transfected with the pBcl-2-Luc reporter (200 ng) along with 300 ng empty vector (Vec), constitutive active AKT (AKT-CA), or dominant negative AKT (AKT-DN). Cells were treated with E₂ (1 nM), ICI 182,780 (100 nM), or E₂ + ICI and were harvested 24 h later and assayed for luciferase activity. Data are represented as fold change normalized to vehicle-treated Vec control, and the values are the means and standard errors of double treatments from a single experiment, and representative of at least two independent experiments. Asterisk denotes statistical significance from vector control ($p < 0.05$). (B) MCF-7 cells were transfected with 5 μ g empty vector (Vec) or constitutive active AKT (AKT-CA) and were treated with vehicle control (Con) or E₂ (1 nM) with or without ICI 182,780 (100 nM), and harvested 24 h later. Fifty micrograms whole cell extracts were subjected to Western blot analysis using a Bcl-2 antibody. The blots were then stripped and re-probed with an actin antibody as an internal loading control. Data are represented as Bcl-2 protein level relative

to actin (Bcl2/actin) and normalized to vehicle-treated Vec control. The blot shown is representative of at least three independent experiments. (C) Densitometry of Western blot analysis from 2B. Asterisk denotes statistical significance from vector control ($p < 0.05$).

**Figure 3.**

AKT differentially potentiates ER α and ER β activity at the Bcl-2 promoter. (A) HEK 293 cells were transfected with the pBcl-2-Luc reporter (200 ng), along with 300 ng empty vector (Vec) or constitutive active AKT (AKT-CA), along with either 100 ng ER α or ER β . Cells were treated with vehicle control (Veh) or E₂ (1 nM) and were harvested after 24 h of treatment and assayed for luciferase activity. Data are represented as RLU normalized to vehicle-treated Vec control, and the values are the means and standard errors of double treatments from a single experiment and are representative of at least two independent experiments. Asterisk denotes statistical significance from vector control ($p < 0.05$). (B) HEK 293 cells were transfected as in (A) with the addition of ER domain mutants ER α -AF-I and ER-AF-II. The experiment was carried out as in (A). Asterisk denotes statistical significance from vector control of each ER construct ($p < 0.05$). (C) HEK 293 cells were transfected with a total of 5 μ g DNA of empty vector (Vec) or ER α , with or without constitutive active AKT (AKT-CA) and were treated with vehicle control (Con) or E₂ (1 nM), and harvested 24 h later. Whole cell extracts (50 μ g) were subjected to Western blot analysis using Bcl-2 antibody. The blots were then stripped and re-probed with actin antibody as an internal loading control. Data are represented as Bcl-2 protein level relative to actin (Bcl2/actin) and normalized to vehicle-treated Vec control. (D) Densitometry of the Western blot analysis from E. The blot shown is representative and the data are the results of at least three independent experiments. Asterisk denotes statistical significance from vector control ($p < 0.05$).

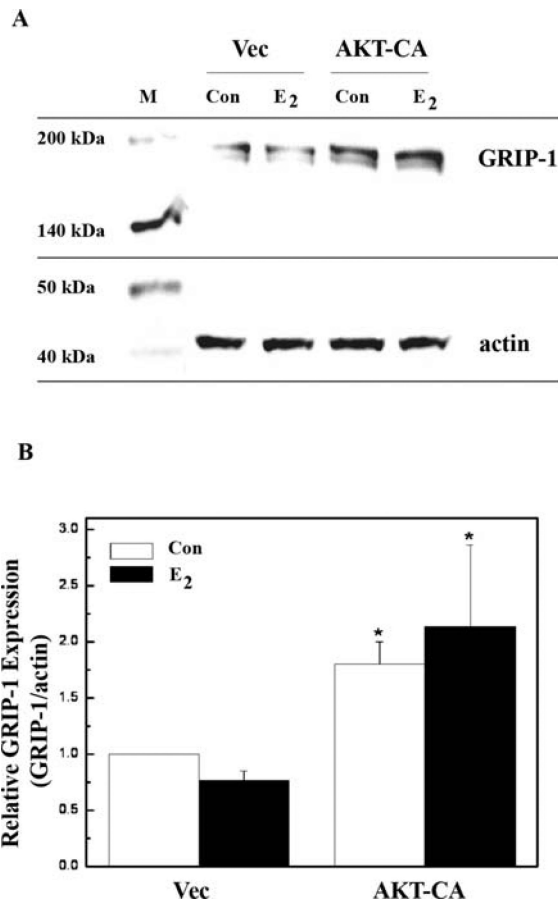


Figure 4.

AKT enhances GRIP1 protein expression. (A) MCF-7 cells were transfected with 5 μ g empty vector (Vec) or constitutive active AKT (AKTCA) and were treated with either vehicle (con) or E₂ (1 nM) for 24 h. Once harvested, 50 μ g whole cell extracts were subjected to Western blot analysis using a GRIP1 antibody. The blots were then stripped and re-probed with an actin antibody as an internal loading control. (B) Densitometry of the Western blot analysis. Data are represented as GRIP1 protein level relative to actin (GRIP1/actin) and are normalized to vehicle-treated Vec control and are the results of three independent experiments. Asterisk denotes statistical significance from vector control ($p < 0.05$).

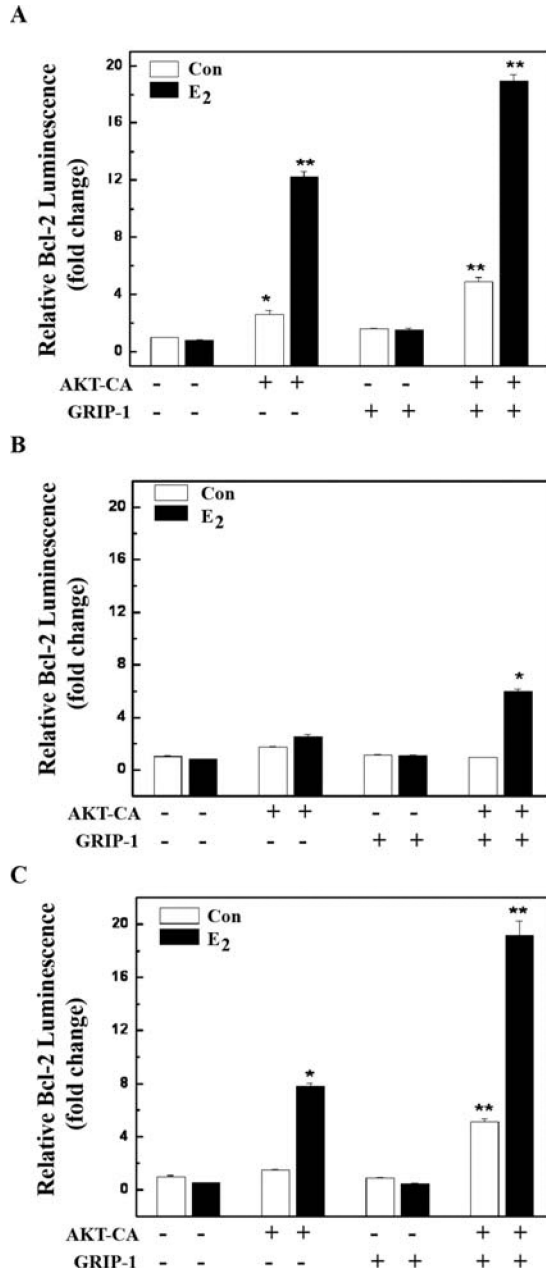


Figure 5.

AKT and GRIP1 cooperatively enhance ER α -mediated Bcl-2 promoter activation. (A) HEK 293 cells were transfected with a pBcl-2-Luc reporter, along with constitutive active AKT (AKT-CA) or GRIP1 and ER α (full length). Cells were treated with vehicle control (Con) or E₂ (1 nM) and were harvested after 24 h of treatment for measurement of luciferase activity. Data are represented as fold change and are normalized to vehicle-treated Vec control. All the values are the means and standard errors of double treatments from a single experiment, and are representative of at least two independent experiments. Asterisk denotes statistical significance from vector control cells. Two asterisks denotes statistical significance from AKT-CA control cells. (B) Experimental design the same as in (A) except ER-AF-I domain mutant was used. (C) Experimental design the same as in (A) except ER-AF-II domain mutant was used.

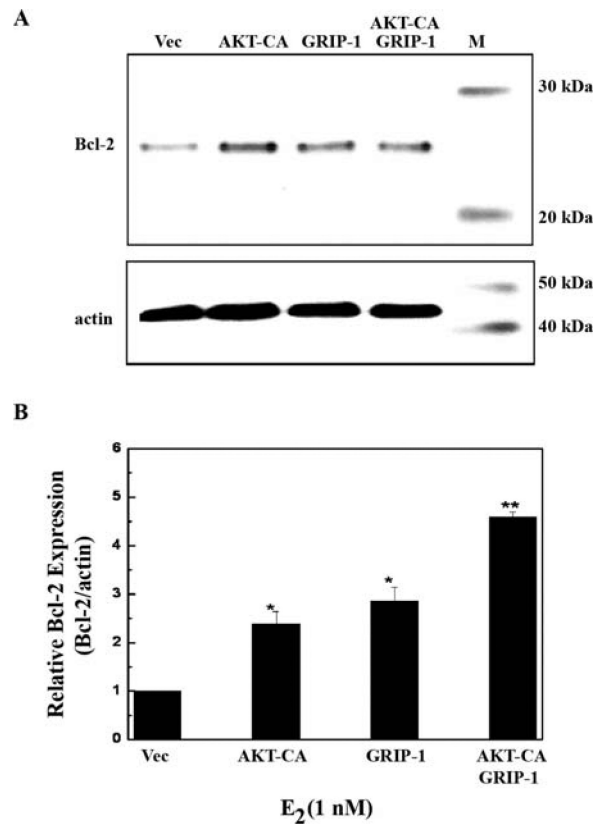


Figure 6. AKT and GRIP1 cooperatively enhance Bcl-2 protein expression. (A) MCF-7 cells were transfected with 5 μ g vector control (Vec), constitutive active AKT (AKT-CA), pSG5-GRIP1-HA (GRIP1), or 2.5 μ g each AKT-CA and GRIP1. Cells were treated with E₂ (1 nM) for 24 h prior to harvesting. Whole cell extracts (50 μ g) were subjected to Western blot analysis using a Bcl-2 antibody. The blots were then stripped and re-probed with an actin antibody as an internal loading control. (B) Densitometry of the Western blot analysis. Data are represented as Bcl-2 protein level relative to actin (Bcl2/actin) and are normalized to vector control. Data are the results of two independent experiments. Asterisk denotes statistical significance from vector E₂, and two asterisks denotes statistical significance from AKTCA E₂ and GRIP-1 E₂ (p<0.05).