Estradiol Abrogates Apoptosis in Breast Cancer Cells through Inactivation of BAD: Ras-dependent Nongenomic Pathways Requiring Signaling through ERK and Akt[□]

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Estrogens such as 17- β estradiol (E_2) play a critical role in sporadic breast cancer progression and decrease apoptosis in breast cancer cells. Our studies using estrogen receptor-positive MCF7 cells show that E_2 abrogates apoptosis possibly through phosphorylation/inactivation of the proapoptotic protein BAD, which was rapidly phosphorylated at S112 and S136. Inhibition of BAD protein expression with specific antisense oligonucleotides reduced the effectiveness of tumor necrosis factor- α , H_2O_2 , and serum starvation in causing apoptosis. Furthermore, the ability of E_2 to prevent tumor necrosis factor- α -induced apoptosis was blocked by overexpression of the BAD S112A/S136A mutant but not the wild-type BAD. BAD S112A/S136A, which lacks phosphorylation sites for p90^{RSK1} and Akt, was not phosphorylated in response to E_2 in vitro. E_2 treatment rapidly activated phosphatidylinositol 3-kinase (PI-3K)/Akt and p90^{RSK1} to an extent similar to insulin-like growth factor-1 treatment. In agreement with p90^{RSK1} activation, E_2 also rapidly activated extracellular signal-regulated kinase, and this activity was down-regulated by chemical and biological inhibition of PI-3K suggestive of cross talk between signaling pathways responding to E_2 . Dominant negative Ras blocked E_2 -induced BAD phosphorylation and the Raf-activator RasV12T35S induced BAD phosphorylation as well as enhanced E_2 -induced phosphorylation at S112. Chemical inhibition of PI-3K and mitogen-activated protein kinase kinase 1 inhibited E_2 -induced BAD phosphorylation at S112 and S136 and expression of dominant negative Ras-induced apoptosis in proliferating cells. Together, these data demonstrate a new nongenomic mechanism by which E_2 prevents apoptosis.

INTRODUCTION

The growth of human and animal tumors is a balance between cellular proliferation and death. Cell death in tumors occurs through necrosis and apoptosis, and highly malignant tumors often have high rates of both apoptosis and necrosis (Gompel *et al.*, 2000). Thus, it is not surprising that mitogenic substances such as insulin-like growth factor (IGF-1) also serve as cellular survival factors and are able to decrease rates of apoptosis. Estrogens, in particular 17- β estradiol (E_2), are well-characterized mitogens for mammary tissues and epithelial cells of the female reproductive tract. Whereas estrogens exert antiapoptotic influence in neurons (Zhang *et al.*, 2001), epithelial cells of the female reproductive tract (Leung and Wang, 1999; Choi *et al.*, 2001), immune system, blood cells, and endothelial cells (Bynoe *et al.*, 2000; Haynes *et al.*, 2000), they also have significant apoptotic effects on certain classes of bone-derived cells (Hughes *et al.*, 1996; Kameda *et al.*, 1997) and some immune system cells (Zajchowski and Hoffman-Goetz, 2000; Okasha *et al.*, 2001).

Furthermore, in some estrogen receptor (ER)-negative cells, overexpression of ER often leads to apoptosis (Kushner *et al.*, 1990) perhaps through activation of p38^{MAPK} (Srivastava *et al.*, 1999, and references therein). Whereas E_2 promotes cell survival in ER-positive cells both in cell culture and in xenograft models, antiestrogens induce apoptosis in both ER-positive and ER-negative cells (Srivastava *et al.*, 1999; Chen *et al.*, 2000; Gandhi *et al.*, 2000). Whether the latter results can be explained by expression of ER at low levels or an isoform of ER different from ER α is presently unknown.

In the ER-positive MCF7 breast cancer cell line, antiapoptotic effects of E_2 have been reported previously (Huang, 1997; Perillo *et al.*, 2000; Ahamed *et al.*, 2001); however, the mechanisms of action are poorly defined. It is now established that E_2 induces transcription of the BCL2 gene and increases expression of BCL2 protein, which exerts antiapoptotic action in many cell types (Huang, 1997; Dong *et al.*, 1999; Leung and Wang, 1999). In addition to genomic actions, E_2 has been known to exert rapid, likely nongenomic actions both in the whole animal and in cultured cells (Szego, 1974; Huang, 1997; Razandi *et al.*, 2000a,b). Previously, we had observed that estrogens require the function of the Ras, mitogen-activated protein kinase kinase (MEK)1 and extracellular signal-regulated kinase (ERK) pathway to induce G1/S phase transition (Ahamed *et al.*, 2001) and elicit down-regulation of the Cdk2 inhibitor p27^{kip1} (Foster, 2003; Zhu *et al.*, 2003). Given that E_2 is now known to activate kinase-regulated signal transduction pathways in MCF7 and other cells (Migliaccio *et al.*, 1996, and references therein;

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Abbreviations used: Dm, double mutant; DN, dominant negative; Wt, wild type.

Singh *et al.*, 2000; Zhang and Shapiro, 2000), we have examined potential nongenomic pathways involved in its antiapoptotic actions, in particular the phosphatidylinositol 3-kinase (PI-3K)/Akt/BAD pathway, which mediates BAD inactivation and favors cell survival. In this article, we demonstrate for the first time that E₂ induces BAD phosphorylation through Ras/PI-3K/Akt as well as Ras/ERK/p90^{RSK1} pathways, and we also show that the function of the PI-3K/Akt pathway is required for E₂ to block apoptosis induced by tumor necrosis factor (TNF- α), H₂O₂, and serum withdrawal. Our data further provide evidence that BAD function is required for induction of apoptosis in MCF7 cells by TNF- α , H₂O₂, and serum withdrawal and that overexpression of the dual phosphorylation site mutant of BAD, but not the wild type, abolished the antiapoptotic actions of E₂.

MATERIALS AND METHODS

Cell Culture, Serum Starvation, Treatment with E₂ and Other Agents

MCF7 cells (a gift from R.P. Shiu; Dubik and Shiu, 1992) were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum, penicillin G, and streptomycin at 37°C in 5% CO₂. Cells were kept in 1% serum for 24 h in DMEM (phenol red [PR]-free) medium and completely serum withdrawn for additional 24–48 h before the experiments. Before treatments, cells were washed once with warm phosphate-buffered saline and treated in medium free of serum and PR. LY294002 (5 μ M in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium [MTT] assay, 20 μ M in other assays) (Sigma-Aldrich) and ICI182,780 (1 μ M), PD98059 (50 μ M) were added 1 h before E₂ (1 nM in MTT assay and 10 nM in other assays) or IGF-1 (10 ng/ml). ICI182,780 is a gift from Zeneca Pharmaceuticals (Wilmington, DE). Where appropriate, cells were treated with dimethyl sulfoxide or ethanol as controls.

Cell Survival Assay

At 20–30% confluence, cells were treated in triplicates in 24-well plates. At 24-h intervals, MTT assay was performed according to the manufacturer's (Sigma-Aldrich) protocol. Briefly, MTT (5 mg/ml) was added to equal 1/10 the culture volume and incubated for 3 h. Medium was removed, and the converted dye was solubilized in ice-cold isopropanol. Absorbency of dye was measured at 560 nm with background subtraction at 630 nm on a microplate reader (EL 340 Bio Kinetics Reader; Bio-Tek Instruments, Winooski, VT).

DNA Fragmentation Assay (Cell Death Detection ELISA^{PLUS})

Apoptosis was induced with H₂O₂ (2 mM) for 1.5 h, TNF- α (10 ng/ml) for 8 h, or by complete removal of growth factors for 72 h. Cells were pretreated with E₂ or IGF-1 for 1 h before apoptotic stimuli. DNA fragmentation was quantified using enzyme-linked immunosorbent assay (ELISA) kit (Cell Death ELISA; Roche Diagnostics, Indianapolis, IN) with slight modification to the manufacturer's protocol. Briefly, 50,000 cells of each treatment were lysed in 100 μ l of lysis buffer, and a fraction of the supernatant was subjected to reaction for 2 h with the immunocomplex of anti-DNA conjugated with peroxidase, which binds to nucleosomal DNA, and antihistone-biotin, which interacts with streptavidin-coated wells in a microtiter plate. At the end of the incubation, substrate was added, and color development was quantified at 405-nm wavelength.

Adenoviral Infections

PTEN (kindly donated by Dr. R Bookstein; Cheney *et al.*, 1999), DNAAkt (kindly donated by Dr. K Walsh; Kureishi *et al.*, 2000), and LacZ (Q.Biogene, Carlsbad, CA) were transduced at 60, 30, and 30 multiplicity of infection (MOI), respectively. Optimum MOIs were determined by analyzing DNA profiles (by flow cytometry) in response to virus dosage. To study DNA fragmentation with TNF- α or H₂O₂, cells were maintained in DMEM (PR free) with 2.5% fetal bovine serum for 24 h after infection followed by induction of apoptosis. In 2A, cells were completely serum withdrawn for additional 48 h after infection.

Western Blot Analysis

Cells were washed once with ice-cold phosphate-buffered saline and lysed with buffer containing 50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 mM NaVO₃, 1 mM NaF.

For the detection of poly(ADP-ribose) polymerase (PARP) cleavage, cells were lysed in ice-cold buffer containing 50 mM Tris-HCl, 6.5 M urea, 5% mercaptoethanol, 2% SDS, and protease inhibitors, sonicated for 20 s, and briefly centrifuged to remove cell debris (Xu *et al.*, 2002).

For cytochrome *c* detection, cytosolic fraction was prepared as described in Hirai and Wang (2001) with some modifications. Cells were suspended in hypotonic buffer containing 3.3 mM HEPES, pH 7.5, 1.7 mM KCl, 0.25 mM MgCl₂, 0.17 mM EGTA, and 0.17 mM EDTA, 6.6 μ g/ml leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, 16.7 mM NaF, 66.7 μ M Na₃VO₄, and 8.3 mM sodium β -glycerophosphate. After incubation on ice for 30 min, cells were homogenized with a Dounce homogenizer and centrifuged at 1000 $\times$ g for 5 min at 4°C to discard unbroken cells and nuclei. The resulting supernatant was centrifuged at 10,000 $\times$ g for 10 min at 4°C to obtain the heavy-membrane fraction (pellet) and the cytosol (supernatant).

Aliquots of cell extracts containing 50–100 μ g of total proteins or immunoprecipitates of desired proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes, and probed with specific antibodies to phospho-ERKs [pTepY] (Promega, Madison, WI), phospho-BAD [S112 and S136] (Upstate Biotechnology, Lake Placid, NY), ERK2, PTEN, BAD, PARP, cytochrome *c*, Ras, and Akt (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Required concentrations of antibodies for Western blot analyses were determined using manufacturers' protocols. Proteins were detected by ECL (Amersham Biosciences, Piscataway, NJ). The band intensities/fold activities were measured by Sigma Gel software (Jandel Scientific, Winooski, VT).

Immunocomplex Kinase Activity Assays

Antibodies against ERK2, Akt, GSK3 β , p85 (PI-3K), and protein A/G plus beads were purchased from Santa Cruz Biotechnology. Anti p90^{RSK1} antibody, soluble BAD, and IRS-1 were purchased from Upstate Biotechnology. Myelin basic protein (MBP) was from Invitrogen (Carlsbad, CA). One hundred to 200 μ g of total protein was immunoprecipitated with antibody in excess and protein A/G plus beads at 4°C overnight. Precipitates were washed three times with TG buffer (250 mM NaCl, 20 mM Tris, 0.5% NP-40) and once with kinase buffer (50 mM HEPES, pH 7.4, 15 mM MgCl₂). Kinase reactions were performed by incubating immunoprecipitates and the specific substrates (0.2–1 μ g/sample) in kinase mixture (20 μ M ATP, 5 μ Ci of [γ -³²P]ATP, 1 mM dithiothreitol, 0.1 mM Na₃VO₄ in kinase buffer) for 30 min at room temperature. Reactions were stopped with 4 $\times$ Laemmli's sample buffer and were resolved on SDS-PAGE. Band intensities of phosphorylated substrates in autoradiograms were measured by densitometry.

Phosphorylation Assay (Nonradioactive) with BAD Mutants

p-GEX-BAD^{wt/mutant} (kindly donated by Drs. P Cohen; Lizcano *et al.*, 2000) and AM Tolkovsky; Virdee *et al.*, 2000) were grown in *Escherichia coli*, and proteins were isolated using glutathione-agarose beads (Sigma-Aldrich) followed by elution in the presence of reduced glutathione. Ten micrograms of BAD protein was incubated with 75 μ g of whole cell lysate in 50 μ l of kinase buffer in the presence of 25 μ M ATP for 30 min. At the end of the reaction, 15 μ l of agarose-conjugated glutathione was added and shaken for 1 h at 40°C. Beads were collected, washed three times with TG buffer, and resuspended in 25 μ l of 2 $\times$ Laemmli's sample buffer.

Treatment with BAD Oligonucleotides

BAD antisense oligonucleotides (with phosphorothioate linkages) were synthesized and purified by Sigma-Genosys (Woodlands, TX). Cells were transfected twice with LipofectAMINE Plus reagent according to the manufacturer's (Invitrogen) protocol 48 h apart. Transfections were carried out in six-well plates with 1 μ g of oligonucleotide per well. Twenty-four hours after the second transfection, cells were treated and collected for desired analyses. Antisense oligonucleotides used in this study encoded complementary sequences to the coding regions including translation initiation (BAD antisense 1) and termination codon (BAD antisense 2) sites for the human BAD gene. Oligonucleotide sequences used were BAD antisense 1, 5'-TGGGATCTG-TAACATGCT-3'; BAD antisense 1 (scrambled/control), 5'-GGTTAGGT-CAATTACTGCT-3'; and BAD antisense 2, 5'-CGAAGGTCACCTGGGA-3'. Cells were treated with H₂O₂ or TNF- α (with or without E₂) or completely serum withdrawn for 72 h to induce apoptosis. DNA content was analyzed by flow cytometry and sub-G₁ population was considered as apoptotic (Ormerod *et al.*, 1992).

Transient Transfection, Microscopic Analysis of Nuclear Morphology, and Confocal Microscopy

MCF7 cells grown on coverslips were transiently transfected with FLAG-tagged BAD mutants and enhanced green fluorescent protein (EGFP, 15% of total DNA) by using LipofectAMINE Plus reagent according to the manufacturer's (Invitrogen) protocol. The BAD constructs used in this study were human BAD wild-type (Wt) and human BAD serine75 and serine99 (equivalent to mouse serine112 and serine136, respectively) mutated to alanine (Dm)

and were kindly donated by Dr. H.G. Wang (Hirai and Wang, 2001). Coverslips were fixed in 3% paraformaldehyde and mounted on to glass slides. Nuclei were stained with 3 μ g/ml Hoechst 33342 (Sigma-Aldrich) and analyzed for the blue (DNA) and green (EGFP) fluorescence by using a fluorescence microscope (Leitz DMRB) or the confocal microscope. Cells expressing EGFP were considered as coexpressing BAD mutants. We have previously validated the coexpression of plasmid constructs in MCF7 cells (Foster, 2003). Morphological changes in the nuclei were analyzed and condensed/shrunk nuclei were counted as apoptotic as opposed to normal round nuclei (Candolfi *et al.*, 2002).

FLAG-wtBAD and RasN17, RasV12T35S (kindly donated by Drs. G.L. Johnson and C.M. Counter (Hamad *et al.*, 2002, respectively) or empty vector were cotransfected at 1:1 ratio for coexpression studies. For macroscopic analyses, cells grown on coverslips were analyzed with Ras or empty vector constructs together with EGFP (15% of total DNA) and processed and analyzed as described above.

Statistical Analysis

Statistical analysis was performed using Student's *t* test (Prism; GraphPad Software, San Diego, CA), and $p \leq 0.05$ is considered significant.

RESULTS

Inhibition of Apoptosis in MCF7 Cells by E_2 Is Dependent on the PI-3K/Akt Pathway

Previous work in our laboratory has shown that estrogens decreased cell death and apoptosis induced in MCF7 cells by mitogen withdrawal (Ahamed *et al.*, 2001). Recent work indicates that induction of cell growth by E_2 in MCF7 cells may require activity of the PI-3K pathway (Lobenhofer *et al.*, 2000) and the Ras/ERK pathway (Castoria *et al.*, 2001). Furthermore, we reported that G1/S phase transition and Cdk2 activation induced by estrogens is inhibited by dominant-negative (DN) Ras mutants (Ahamed *et al.*, 2001; Foster, 2003; Zhu *et al.*, 2003), and the MEK1 inhibitor PD98059 abrogated the increase in cyclin D1 accumulation resulting from estrogen treatment (Ahamed *et al.*, 2001).

Given this background, we sought to determine whether PI-3K plays an important role in estrogen action with respect to cell growth and apoptosis. In preliminary experiments (our unpublished data), we verified that E_2 promoted cell survival/growth under low serum conditions and further established that the PI-3K inhibitor wortmannin inhibited this effect of E_2 . To confirm the requirement of PI-3K activity for the cell survival induced by E_2 , MCF7 cells were treated with E_2 in the presence or absence of LY294002, a specific PI-3K inhibitor (Sanchez-Margalet *et al.*, 1994) in serum-free medium. In the absence of E_2 , there was significant cell loss reflected in MTT uptake over a 5-d period ($p < 0.05$). E_2 completely blocked this decrease and maintained MTT uptake at or above day 1 level (Figure 1A). Treatment with LY294002 abolished the ability of E_2 to increase cell numbers as indicated by MTT uptake (day 2) or maintain cell survival (day 4, 5) and also reduced viability in the control cultures at day 4 and 5. These results suggest that survival of MCF7 cells under conditions of serum withdrawal requires PI-3K activity and that the ability of E_2 to protect cells from death as well as to induce growth is blocked by abrogation of PI-3K activity. MTT results simply demonstrate the amount of live cells present under given conditions and, in the case of E_2 , this is may present the sum of antiapoptotic and mitogenic effects.

The above-mentioned results and prior observations suggest that E_2 may decrease apoptosis in MCF7 cells. Because TNF- α induces apoptosis through activation of caspases 3, 6, 7, and 8 and/or JNK activation (Budihardjo *et al.*, 1999) in a variety of cell types, including MCF7 cells (Burow *et al.*, 1999; Guicciardi *et al.*, 2000; Tang *et al.*, 2001), we treated MCF7 cells with TNF- α in the presence of E_2 , 17- α estradiol (an estrogen of low potency), E_2 plus an excess of the specific

E_2 antagonist ICI182,780. TNF- α increased apoptosis as measured by DNA fragmentation by four- to fivefold, and E_2 was able to reduce this effect significantly, whereas 17- α -estradiol had no protective effect. The presence of an excess of the E_2 antagonist blocked the protective effect of E_2 in TNF- α -treated cells (Figure 1B). PARP cleavage, a well-known late event in the apoptosis process (Duriez and Shah, 1997), was induced by TNF- α , and this cleavage was significantly blocked by E_2 pretreatment (Figure 1C).

The reactive oxygen species H_2O_2 is known to induce apoptosis in a variety of cell types, including MCF7 cells (Kim *et al.*, 2000), and the apoptotic effect of H_2O_2 in these cells is decreased by pretreatment with E_2 or overexpression of BCL2 in a synergistic manner (Perillo *et al.*, 2000). Furthermore, E_2 and overexpression of BCL2 exhibit additive inhibitory effects on cleavage of PARP (Duriez and Shah, 1997) induced by H_2O_2 . In confirmation of these data, we found that E_2 pretreatment can block DNA fragmentation as well as PARP cleavage induced by 2 mM H_2O_2 (Figure 1, B and C). Pretreatment with 17- α estradiol had no significant protective effect against H_2O_2 , suggesting that E_2 effects are unlikely to be due to its potential free radical scavenger function (Behl *et al.*, 1997).

Apoptotic effects of certain stimuli, including those activating the extrinsic pathway, are manifested at least in part via their effects on mitochondrial membrane integrity (Downward, 1999; Gross *et al.*, 1999). Release of cytochrome *c* from dysfunctional mitochondria contributes to the formation of the apoptosome, which ultimately activates the downstream caspase pathways (Budihardjo *et al.*, 1999). Because the results presented in Figure 1, A–C, would predict that E_2 may preserve mitochondrial integrity in the presence of apoptotic stimuli, we sought to determine whether E_2 would preserve mitochondrial integrity by assessing cytoplasmic cytochrome *c* protein level (Figure 1D). In untreated or E_2 only-treated cells, cytoplasmic cytochrome *c* was undetectable. As expected, H_2O_2 , TNF- α , and serum withdrawal (see below) increased cytochrome *c* in the cytoplasm. Cotreatment with E_2 decreased cytochrome *c* release induced by TNF- α to a similar extent as that observed in cells cotreated with serum and TNF- α . Together, these results suggest that E_2 protects cells against apoptosis induced by TNF- α , H_2O_2 , and serum withdrawal (our unpublished data), possibly by maintaining mitochondrial integrity.

Estradiol Effects on H_2O_2 -, TNF- α -, and Serum Withdrawal-induced Apoptosis Are Abolished by Dominant Negative Akt (DNakt) and Wild-Type PTEN

To establish the role of the PI-3K pathway in E_2 -induced antiapoptosis, we used adenoviral vectors to interfere with operation of this pathway. Cells were transduced with adenoviral wild-type PTEN/MMAC (Figure 2D) that effectively decreases activity of the PI-3K pathway by hydrolysis of PI-3K products (Maehama and Dixon, 1999), and overexpression of PTEN has been used to decrease PI-3K products in other studies (Nakashima *et al.*, 2000). As depicted in Figure 2, overexpression of PTEN completely blocked the ability of E_2 or IGF-1 to prevent apoptosis induced by serum withdrawal (A), H_2O_2 (B), and TNF- α (C) compared with adenoviral LacZ (control)-infected cultures. Akt/PKB is a downstream target of PI-3K pathway that promotes cell survival in a number of cell types (Downward, 1998; Datta *et al.*, 1999). Overexpression of DNakt (Figure 2D) enhanced apoptosis induced by the agents and completely reversed the effects of either IGF-1 or E_2 on apoptosis prevention (Figure 2, A–C). These data together with data derived using chemical inhibitors (Figure 1A) strongly suggest that PI-3K/

Figure 1. Inhibition of cell death in MCF7 cells by E₂. (A) MCF7 cells were serum deprived after 20% confluence and treated in PR-free medium with indicated stimuli. LY294002 (5 μ M) was added 30 min before E₂ (1 nM) treatment. MTT uptake was measured at 24-h intervals in four replicates for each treatment. Results are presented as the percentages compared with day 0 (MTT uptake taken as 100%), and data represent the mean from three independent experiments. E₂ treatment groups are significantly different from control, LY294002, or LY294002 + E₂ at days 2, 4, and 5 ($p \leq 0.05$). (B) Apoptosis was induced by TNF- α (10 ng/ml) for 8 h or H₂O₂ (2 mM) for 1.5 h. Cells were pretreated with E₂ (10 nM) with/without 100-fold excess ICI 182,780, 17- α estradiol (10 nM) for 1 h before addition of apoptotic stimuli. DNA fragmentation was assayed using Cell Death ELISA (see MATERIALS AND METHODS), and data represent the mean of three independent experiments with triplicates per experiment for each treatment. Results are expressed as fold changes of apoptosis compared with untreated controls. The percentage of apoptotic cells in the untreated cultures is 2–5% as measured by this and other methods such as Hoechst 33342 staining for DNA and microscopic analysis of cell morphology. The ICI + E₂ + TNF- α group is not different from TNF- α only group ($p > 0.05$). (** $p < 0.005$, * $p < 0.05$). (C) Seventy-five micrograms of cell lysate protein was resolved on SDS-PAGE and Western blotted with anti-PARP antibody that recognizes parent and cleaved bands at 116 and 85 kDa, respectively. Actin levels are shown as protein loading control. Shown is a representative autoradiogram of three independent experiments that produced comparable results. (D) One hundred micrograms of cytoplasmic protein of cells treated with indicated agents were subjected to Western blotting with cytochrome c and actin antibodies. Experiment was repeated two to three times with comparable results.

Akt pathway is essential for the antiapoptotic effects of E₂ or IGF-1 in MCF7 cells. We repeatedly observed that DNA fragmentation (measured by the Cell Death ELISA) in viral-transduced cells was below the levels observed in parental cells (1.8–2.5 vs. 4–5 fold, compare Figures 1B and 2), which lead to an apparent decrease in the magnitude of apoptosis as measured by the Cell Death ELISA in Figure 2.

Estradiol Induces BAD Phosphorylation

BAD is a proapoptotic BH3 domain-containing protein (Gross *et al.*, 1999; Adams and Cory, 2001), which forms heterodimers with BCL2 or BCLx1 (Condorelli *et al.*, 2001) resulting in cytochrome c release from mitochondria. Antiapoptotic agents such as IGF-1 induce BAD phosphorylation at specific serine residues, and phosphorylated BAD is sequestered away from its site of action in the mitochondria by binding to cytosolic 14-3-3 proteins (Datta *et al.*, 1997). Because BAD is phosphorylated on serine 136 (S136) by Akt (Datta *et al.*, 1997), we ascertained the ability of E₂ to induce phosphorylation of BAD at specific residues by using BAD phosphorylation site mutants as in vitro substrates (Figure

3A). Clearly E₂- or IGF-1-treated cell extracts were unable to induce phosphorylation of BAD at S112 in the serine-to-alanine mutant (S112A), whereas S136 phosphorylation was enhanced three- to fourfold. Similar specificity was observed when the S136A BAD mutant was used as the substrate (Figure 3A, right). When the double phosphorylation site mutant (DM) was used as a substrate, only a small fraction of the phosphorylation that was observed with single mutants was detectable. Multiple immunoreactive bands have been previously observed when recombinant BAD was used as a substrate (Datta *et al.*, 1997).

We also measured BAD phosphorylation in vivo by Western blot analysis. After treatment with E₂ or IGF-1 for 15 min, both S112 and S136 phosphorylations were enhanced (>3-fold), and total BAD levels were unchanged in this time frame (Figure 3B).

Because our results demonstrated that E₂ can induce BAD phosphorylation in vitro and in vivo and can prevent cell death in response to several stimuli, we studied the possible changes in phosphorylation status of BAD during apoptosis induction. E₂ increased endogenous BAD phosphorylation

Figure 2. Inhibition of apoptosis in MCF7 cells by E₂ is dependent on the PI-3K/Akt pathway. Viral vectors were used to overexpress PTEN, DN Akt, and Lac-Z (control) at predetermined MOIs. Forty-eight hours after the infection apoptosis was induced by complete serum withdrawal for additional 48 h (A), with H₂O₂ (2 mM) for 1.5 h (B), and with TNF- α (10 ng/ml) for 8 h (C). Where indicated, cells were pretreated with E₂ (10 nM) or IGF-1 (10 ng/ml) for 1 h before apoptotic stimuli. DNA fragmentation was measured as in Figure 1B. Results are expressed as fold change in apoptosis compared with control (untreated) for each infection and represent values of at least three independent experiments with three replicates for each treatment per experiment. In Lac-Z group, serum-starved sample is different from Lac-Z + E₂ at $p < 0.005$ (**); Lac-Z + E₂ is different from PTEN + E₂ or DN Akt + E₂ at $p < 0.005$ (*). In Lac-Z group, H₂O₂-treated sample is different from Lac-Z + E₂ at $p < 0.05$; Lac-Z + E₂ is different from PTEN + E₂ and DN Akt + E₂ at $p < 0.005$. In Lac-Z group, TNF- α -treated sample is different from Lac-Z + E₂ at $p < 0.005$; Lac-Z + E₂ is different from PTEN + E₂ and DN Akt + E₂ at $p < 0.05$ and $p < 0.005$, respectively. (D) Demonstrates overexpression of PTEN and DN Akt by Western blot analysis.

at S112 and S136 above the basal level severalfold, whereas TNF- α and H₂O₂ decreased it below control level (Figure 3C). Cotreatment with E₂ and H₂O₂ or TNF- α restored the BAD phosphorylation to above-control levels. Together, these data strongly suggest that E₂ as well as IGF-1 exert antiapoptotic actions partly through phosphorylation and inactivation of the proapoptotic BAD protein and that phosphorylation of BAD is a consequence of activation of Akt and possibly ERK/p90^{RSK1} because S112 is a target site for p90^{RSK1} (Downward, 1999).

Inhibition of BAD Is a Critical Event in Estradiol-induced Antiapoptosis in MCF7 Cells

Because the effects of apoptotic agents on decreasing BAD phosphorylation (inactivation) were largely reversed by E₂,

BAD inactivation may be a potential nongenomic effect of E₂, which results in cell survival. To further understand the involvement of BAD in cell death and survival in MCF7 cells, we used antisense oligonucleotides to prevent the translation of BAD mRNA. The antisense sequences, BAD antisense 1 (BAD As 1) containing the complementary sequence to the translation initiation codon and BAD antisense 2 (BAD As 2) containing complementary sequence to the translation termination codon were equally effective in down-regulating BAD protein level (Figure 4A), whereas a scrambled oligonucleotide corresponding to BAD As1 (control oligo) was not. Quantification of DNA by flow-cytometry revealed a large reduction of the sub-G₁ fraction-apoptotic cells (Ormerod *et al.*, 1992) in TNF- α -treated (Figure 4B)

Figure 3. Estradiol induces BAD phosphorylation. (A) Glutathione-tagged BAD proteins (BAD S112A, BAD S136A, and BADS112/136A [DM]) were subjected to in vitro kinase assay for 30 min with extracts from MCF7 cells that were treated with E₂ (10 nM) or IGF-1 (10 ng/ml) for 15 min. Proteins were separated using agarose-conjugated glutathione and resolved on SDS-PAGE. BAD phosphorylation at S112/S136 and total BAD protein levels were measured by Western blot. (B) Cells were treated as in A with E₂ (10 nM) or IGF-1 (10 ng/ml) for 15 min, and endogenous BAD phosphorylation was detected by Western blot by using phospho-specific BAD antibodies for S112 and S136. Same blot was probed with anti-BAD antibody. Fold stimulation (compared with untreated control) was calculated by densitometry, and the graph represents values of three independent experiments. (C) MCF7 cells were treated with indicated stimuli (TNF- α [10 ng/ml] for 8 h or H₂O₂ [2 mM] for 1.5 h) with or without 1-h E₂ pretreatment. Equal amounts of whole cell lysate were subjected to Western blotting with anti-phospho and total BAD antibodies. Change in phosphorylation compared with untreated control was calculated by densitometry. Shown is a representative of two independent experiments with comparable results.

as well as serum-starved (our unpublished data) cells in BAD As1- and BAD As2-treated, but not in control oligonucleotide-treated cells. E₂ did not have an additional effect alone or together with TNF- α , in antisense-treated cells compared with control oligonucleotide treated cells (our unpublished data). These observations suggest a critical role of BAD in apoptosis in MCF7 cells and agree with a previous study where BAD overexpression resulted in apoptosis of mammalian cells (Ottillie *et al.*, 1997; Virdee *et al.*, 2000). Change in apoptosis due to TNF- α was calculated by subtracting sub-G1 fraction of untreated from sub-G1 fraction of TNF- α -treated cells for each oligonucleotide-treated population. Data from three independent experiments are shown in Figure 4C. Introduction of BAD As1 or BAD As2 significantly reduced the magnitude of apoptosis in response to TNF- α (**p* < 0.05), H₂O₂, and complete serum starvation (our unpublished data). We next measured DNA fragmentation in oligonucleotide-treated cells in the presence or absence of TNF- α . TNF- α induced four- to fivefold DNA

fragmentation above the basal level (comparable to Figure 1B) in control oligonucleotide- or LipofectAMINE-treated cells. BAD As1 drastically reduced this effect of TNF- α (***p* < 0.005) and maintained the DNA fragmentation near the basal level (Figure 4D). Similar results were observed using BAD As2 (our unpublished data). These data clearly suggest a critical involvement of BAD in cell survival and death in response to several agents in MCF7 breast cancer epithelial cells and agree with the reduction in basal BAD phosphorylation induced by TNF- α and H₂O₂ in the absence of E₂ (Figure 3C).

To assess the effects on BAD in E₂-induced cell survival and consequent changes in cellular morphology, we transiently expressed wild-type BAD (wtBAD) and dual phosphorylation site mutant of BAD (dmBAD) where S112 and S136 are mutated to unphosphorylatable alanine, together with an EGFP expression vector. After the desired treatments, coverslip cultures were stained with Hoechst 33342 and mounted on to glass slides. As shown in Figure 4E, E₂

reduced the TNF- α -induced apoptosis in wtBAD expressing cells by >50%, and importantly had no protective effect in dmBAD-expressing cells. Pretreatment of E₂ had the same protective effect against TNF- α in cells expressing the control vector pcDNA3 or EGFP alone (our unpublished data). It must be emphasized that the overexpression of dmBAD did not alter the number of apoptotic nuclei compared with those in pcDNA3- or EGFP-transfected cultures. There is an absolute requirement for TNF- α to induce apoptosis in dmBAD as well as wtBAD expressing cells. The magnitude of apoptosis observed with cell counting (~8- to 9-fold in Figure 4E) is somewhat higher than that observed with DNA fragmentation ELISA assay (4- to 5-fold in Figures 1B and 4D) in response to TNF- α . Possible explanation for such differences in magnitude may be the fact that only a fraction of all the cells (i.e., the transfected population) is counted in Figure 4E, whereas in Figures 1B and 4D DNA fragmentation of the whole population is measured.

Similarly transfected and treated cells were examined by the confocal microscopy to further analyze the cellular morphological changes (Supplementary Figure 1). TNF- α -induced cellular shrinkage and atypical structure of nuclei in MCF7 cells as has been described previously for fibroblast (Luschen *et al.*, 2000) and neuronal cells (Candolfi *et al.*, 2002). It was difficult to observe the chromatin clumping (typical for apoptosis) in TNF- α -treated cells due to shrinkage of the nuclei as opposed to this feature readily apparent in paclitaxel-treated cells (our unpublished data). Under these conditions, expression of exogenous wtBAD or most importantly, dmBAD (white arrows in fluorescence images and respective differential interference contrast images) did not enhance basal apoptosis (1 and 4). Treatment with TNF- α in wt and dmBAD-transfected cells clearly induced apoptotic changes (2 and 5). Whereas E₂ was able to impede morphological effects of TNF- α in wtBAD-expressing cells (3), it was unable to counteract the effects of TNF- α in dmBAD-expressing cells (6 and 7). Black arrows in 6 and 7 point to the untransfected cells that preserve the normal morphology. Under these conditions, expression of EGFP alone did not induce morphological changes (white arrows in the insert). These observations further support the proposed crucial role of BAD in MCF7 cells and the critical importance of the two specific phosphorylation sites in BAD for the antiapoptotic effects of E₂. It is very clear that dmBAD by itself does not induce apoptosis in the absence of TNF- α . This suggests that in these mammary carcinoma cells, there are mechanisms to possibly sequester BAD away from mitochondria independent of S112 and S136 phosphorylation and binding to 14-3-3 proteins, thus making dmBAD ineffective as an apoptosis inducer by itself. Antiapoptotic effect of E₂ was further evaluated in cells expressing increasing levels of BAD. MCF7 cells were transfected with 1/4 $\times$, 1/2 $\times$, 1 $\times$, and 2 $\times$ ($\times$ = regular dose = 0.6 μ g of DNA for 10 cm²) of each construct and were treated with TNF- α with or without E₂. DNA fragmentation was assayed as in Figure 4D. As shown in Figure 4F, 1, E₂ reduced apoptosis by 45–60% in the wtBAD-expressing cells, irrespective of the dose, in agreement with Figure 4E. E₂-mediated protection was dampened by the overexpression of dmBAD by 50% or more. A moderate dose dependency in response to dmBAD was observed in TNF- α as well as the E₂ + TNF- α -treated cells (Figure 4F, 3). The values of apoptosis as measured by ELISA in pcDNA3, wtBAD, and dmBAD were unrelated to the dose of DNA. Therefore, when the DNA fragmentation values were averaged for the three types of transfections there was absolutely no difference among the constructs (O.D. values at 405 nm of 0.15, 0.16, and 0.15 for pcDNA3,

wtBAD, and dmBAD, respectively). Increasing doses of wtBAD did not influence the basal apoptosis, TNF- α -induced apoptosis, or the protection offered by E₂ as shown in Figure 4F, 2. Panel 4 demonstrates the fold induction of apoptosis in pcDNA3-expressing cells at regular dose of BAD. In wtBAD-transfected cells (Figure 4F, 1 and 2) E₂ is able to decrease the apoptosis due to TNF- α to the same extent as in untransfected or pcDNA3-transfected cells (Figures 1B and 4F, 4) by 50–60% when measured by the ELISA method. Therefore, untransfected cells are unlikely to influence results in the wtBAD-transfected cells. In contrast, the inability of E₂ to prevent apoptosis in dmBAD mutant transfected cells is likely to be underrated as the ELISA measurement is an average of transfected and untransfected cells. In the latter, E₂ will prevent apoptosis in the majority of the cells, thereby diluting the apoptotic effect of dmBAD-transfected cells. Together, the data in Figure 4, E and F, and Supplementary Figure 1 strongly suggest that dmBAD is not an apoptosis inducer by itself in MCF7 cells. This reinforces our notion that in these cells, TNF- α (and perhaps other apoptotic stimuli) is able to translocate cytoplasmic BAD to its mitochondrial sites of action irrespective of phosphorylation at S112 and S136 residues.

Estradiol-induced BAD Phosphorylation Requires Ras-activated Signaling Pathways, and Ras Function Is Critical for Cell Survival of MCF7 Cells

Previous studies in our laboratory had demonstrated that E₂ induced p27^{kip1} degradation and entry of quiescent MCF7 cells to the cell cycle requires Ras activity (Ahamed *et al.*, 2001, 2002; Foster *et al.*, 2001). Other investigators have demonstrated that E₂ rapidly activated Ras in MCF7 cells (Keshamouni *et al.*, 2002), and recent work in our laboratory (Foster, 2003) demonstrated that Ras activity is required for nuclear export and degradation of p27^{kip1} in response to E₂. The phosphorylation of BAD at S112 and S136 has been proposed to be mediated by ERK/p90^{RSK1} and PI-3K/Akt pathways, respectively, in addition to other mechanisms (Downward, 1999, and references therein). Given the role of Ras discussed above and the evidence that the PI-3K and ERK pathways are activated by E₂ (as discussed below), we used chemical inhibitors LY294002 and PD98059 to inhibit these pathways, respectively, and determined the effects of these treatments on BAD phosphorylation. As shown in Figure 5A, E₂ induced BAD phosphorylation on S136 as well as S112 was completely abrogated by LY294002, supporting the idea of cross talk between the MAPK/p90^{RSK1} and PI-3K/Akt pathways in enhancing the BAD phosphorylation (discussed under Figure 7). Similarly PD98059 effectively reduced the phosphorylation of BAD at S112 as determined by Western blots; however, the MEK inhibitor had considerably less effect on BAD S136 phosphorylation (compare E₂ vs. PD + E₂ on BAD phosphorylation on S136). None of the treatments changed total BAD levels in this time frame.

Because Ras is a well-known upstream regulator of the PI-3K/Akt and MEK/ERK/p90^{RSK1} pathways, we analyzed the effects of inhibition of endogenous Ras by using dominant negative Ras RasN17 (DN Ras) or selectively activating the MEK/ERK pathway with RasV12T35S (Hamad *et al.*, 2002) in transient transfection experiments. FLAG-tagged BAD was cotransfected with control empty vector, RasN17, or RasV12T35S into MCF7 cells. Twenty-four hours after the transfection, cells were serum withdrawn for another 24 h and stimulated with E₂ for 15 min. BAD phosphorylation was assessed by Western blotting. Clearly, the ability of E₂ to increase both S112 and S136 phosphorylation (Figure 5B, 1, lane2) of exogenous as well as endogenous BAD was inhib-

Figure 4. BAD inhibition is a critical event in TNF- α -induced apoptosis and its prevention by E₂ in MCF7 cells. (A) MCF7 cells were treated with BAD antisense (BAD As1 and BAD As2) and control oligonucleotides (see MATERIALS AND METHODS), and protein extracts were subjected to Western blotting with anti-BAD and actin antibodies. BAD expression levels measured by densitometry are shown below the BAD blot. (B) Oligonucleotide-treated cells were stimulated with TNF- α (10 ng/ml) for 8 h as described in MATERIALS AND METHODS. Cells were fixed in 70% ethanol and stained with propidium iodide. DNA content was analyzed by flow cytometry, and sub-G1 population was obtained as a measure of apoptosis. The experiment was repeated three times with comparable results, and representative histograms are shown. The percentage below each histogram indicates the respective sub-G1 (M4) fraction. (C) Results (mean $\pm$ SD) of three experiments as in B are presented in the graph. Change in apoptosis due to TNF- α was calculated by subtracting sub-G1 fraction of the untreated from that of TNF- α -treated sample ($\text{sub-G1}_{\text{TNF}\alpha} - \text{sub-G1}_{\text{untreated}}$) for each oligonucleotide-treated population. (* $p < 0.05$ compared with LipofectAMINE or control oligo-treated samples). (D) Oligonucleotide-treated cells were stimulated with TNF- α (10 ng/ml) for 8 h, and DNA fragmentation was assayed by Cell Death ELISA (see MATERIALS AND METHODS). Data represent mean ($\pm$ SD) O.D. values at 490 nm (which is proportional to DNA fragmentation) of three independent experiments with triplicates for each treatment. (** $p < 0.005$ compared with LipofectAMINE or control oligo-treated samples). (E) MCF7 cells grown on coverslips (two coverslips per treatment) were cotransfected with FLAG-BAD constructs (wild-type [wt] and dual phosphorylation site mutant [dm]) with EGFP. Twenty-four hours after the transfection, cells were treated with TNF- α (10 ng/ml) for 8 h with or without 1-h pretreatment with E₂ (10 nM). Cells were fixed in 3% paraformaldehyde, mounted on to glass slides, and stained with Hoechst 33342. Two hundred cells expressing EGFP were randomly selected from each slide, and nuclear morphology was analyzed. Round nuclei with clear periphery were considered as live and shrunk nuclei were considered as dead (Candolfi *et al.*, 2002). The percentage of apoptotic nuclei in the transfected population is shown on the y-axis. Four independent experiments were carried out to validate data. WtBAD-expressing cells treated with E₂ and TNF- α had significantly less apoptosis than similarly treated dmBAD-expressing cells ($\#p < 0.05$) or TNF- α -treated wtBAD-expressing cells (* $p < 0.05$). (F) Cells expressing FLAG-tagged wtBAD, dmBAD, or empty vector pcDNA3 at indicated doses were treated with TNF- α with or without E₂ and analyzed as in D. In 1, reduction of apoptosis in response to E₂ is graphed against the dose of BAD. The percentage of reduction was calculated by the formula $[100 - (\text{apoptosis}_{\text{TNF}\alpha + \text{E}_2} \div \text{apoptosis}_{\text{TNF}\alpha} \times 100)]$. In 2 and 3, the fold apoptosis compared with untreated control for each transfection is presented. Nonlinear regression curves for data points were drawn using GraphPad Prism software (GraphPad Software). Means of two independent experiments for each dose are presented in the graphs. Panel 4 shows the result of a representative experiment with pcDNA3 at the regular dose. Same treatments with untransfected MCF7 cells show comparable results (Figure 1B).

Figure 4 (continued).

ited by RasN17 (lane 4), which by itself did not regulate BAD phosphorylation (lane 3). The MEK/ERK-selective activator RasV12T35S by itself significantly activated BAD S112 phosphorylation and moderately activated exogenous BAD S136 phosphorylation (lane 5), and E₂ was able to enhance this effect (lane 6). Together, the results in Figure 5, A and B,

strongly indicate that the ability of E₂ to stimulate BAD phosphorylation and thereby prevent apoptosis is mediated through Ras-activated MEK/ERK and PI-3K/Akt pathways.

To directly analyze the apoptotic effects of RasN17 in these cells, we stained nuclei of Ras-transfected cells and studied them by using the confocal microscope. Nuclei cor-

Figure 5. Estradiol-induced BAD phosphorylation is mediated through Ras-activated signaling pathways. (A) MCF7 cells were pretreated with LY294002 (20 μ M) or PD98059 (50 μ M) for 1 h before E₂ treatment for 15 min. BAD phosphorylation at S112 or S136 and total BAD protein level were measured in cell lysates by Western blotting. LY294002 and PD98059 had minimal effects on BAD basal phosphorylation. Whereas LY294002 reversed the phosphorylation at both S112 and S136 residues induced by E₂, PD98059 effectively blocked S112 phosphorylation and partially reduced S136 phosphorylation in response to E₂. Blots shown are from a representative experiment that was repeated at least three times with similar results. (B) 1, FLAG-tagged wtBAD was cotransfected with empty vector pcDNA3, RasN17, or RasV12T35S (selective Raf activator) at 1:1 ratio into MCF7 cells. Twenty-four hours after the transfection, cells were serum withdrawn for additional 24 h and treated with E₂ for 15 min. Cell lysates were analyzed for phosphorylated BAD (endogenous/exogenous), endogenous total BAD, and FLAG by Western blotting. Note that RasN17 abolished E₂-induced BAD phosphorylation and E₂ further enhanced the RasV12T35S-induced BAD phosphorylation at both S112 and S136. Panel 2 shows the Ras levels in untransfected and Ras-overexpressing cells. Blots shown are from a representative experiment that was repeated twice with comparable results. (C) Cells grown on coverslips (as triplicates) were cotransfected with EGFP together with indicated constructs. Cells were maintained in complete medium for 48 h after the transfection, fixed in 3% paraformaldehyde, stained with Hoechst 33342, and mounted on to glass slides. In RasN17-transfected cultures, the floating cells were collected and cyto-spun on to glass slides. At these time points the amount of floating cells observed in other cultures were negligible. Nuclear morphology of cells with green fluorescence was scored either as normal or apoptotic using fluorescence microscopy. In untransfected control cultures, cells were randomly selected for scoring. Two hundred cells/nuclei were counted from each slide. Mean results of two experiments are shown in the graph. Representative images are shown in the supplementary Figure 2. Note that expression of empty vector, EGFP, or RasV12T35 did not increase the number of detached cells.

responding to EGFP-expressing cells (cotransfected with Ras or control vector) were counted, and the graph (Figure 5C) shows the number of fragmented nuclei as a percentage of total number of cells. Transient overexpression of RasN17 induced seven- to eightfold apoptosis over a 48-h period, whereas the expression of RasV12T35S or empty vector had minimum effects on apoptosis observed in cells coexpressing EGFP. Representative morphological data are shown in Supplementary Figure 2 (1–5), which clearly demonstrates that expression of EGFP, empty vector, or RasV12T35S (1–3) did not alter nuclear morphology. However, RasN17-induced apoptotic nuclear morphology in the detached dead cells (5), whereas untransfected attached cells from the same cultures (4) had normal nuclei. These results demonstrate that Ras function is required to protect MCF7 cells from dying even in the presence of growth medium and that this effect may be mediated at least partly by phosphorylating and inactivating proapoptotic BAD. The observation that E₂ induced BAD phosphorylation was abolished by inactivation of endogenous Ras (Figure 5B) further highlights the involvement of Ras-mediated pathways in E₂ signaling and demonstrates that Ras plays a critical role in the antiapoptosis mediated by E₂ in MCF7 breast cancer cells.

Estradiol Activates the PI-3K/Akt Pathway, Leading to Phosphorylation of Downstream Targets

Activation of PI-3K and Akt and Inactivation of GSK3 β by E₂. The above-mentioned data strongly argues that the ability of E₂ to decrease apoptosis in MCF7 cells is at least partly mediated by the PI-3K/Akt pathway. Transient activation (measured by S473 phosphorylation) of Akt by E₂ in MCF7 cells was reported previously (Castoria *et al.*, 2001; Razandi *et al.*, 2002) (see DISCUSSION) while this work was in progress (Fernando and Wimalasena, 2000, 2002). Our data suggest that the PI-3K/Akt pathway may need to be activated for a prolonged period to maintain BAD in an inactivated (phosphorylated) state. Therefore, we measured detailed kinetic behavior of the PI-3K response to E₂. PI-3K activation was determined by phosphorylation of IRS-1 on S612 by in vitro kinase assay using immunoprecipitates of the p85 subunit of PI-3K. As shown in Figure 6A, E₂ was able to rapidly activate PI-3K approximately threefold, similar in magnitude to the activation elicited by IGF-1. A detailed time course of Akt catalytic activity measured by in vitro kinase assays (n = 3) using Akt immunoprecipitates and BAD as the substrate is shown in Figure 6B. Treatment with E₂ does not change protein levels of Akt in this time period (our unpublished data). These data demonstrate that in contrast to the results from the S473 phosphorylation assay (Castoria *et al.*, 2001), catalytic activity of Akt is increased at least for 120 min. GSK3 β is a downstream target of Akt (Cross *et al.*, 1995) and because of its potential role in the cell cycle, we determined GSK3 β activity by in vitro kinase assay using immunoprecipitates of GSK3 β . As shown in Figure 6C, E₂ rapidly and strongly inhibited GSK3 β activity. As expected, another Akt activator IGF-1 also completely inhibited GSK3 β activity. The effect of E₂ was sustained for 60 min (our unpublished data), and such a time course is compatible with the increase in cyclin D1 protein observed 3–4 h after E₂ addition to quiescent cells (Foster and Wimalasena, 1996; Foster *et al.*, 2001).

Activation of ERKs by Estradiol. It is known that p90^{RSK1} is phosphorylated at S364 and T574 residues by ERKs, leading to its activation (Dalby *et al.*, 1998), and our results on BAD phosphorylation imply that E₂ activates p90^{RSK1}. Therefore,

we directly determined that E₂ rapidly activates p90^{RSK1} by in vitro kinase assay using immunoprecipitates of p90^{RSK1} (Figure 6, D and E), which shows that p90^{RSK1} is activated within 5 min by E₂. A number of studies reported that E₂ rapidly activates ERK1 and 2 in MCF7 cells (Improta-Brears *et al.*, 1999, and references therein) and in a number of other cell types (Singh *et al.*, 1999; Kousteni *et al.*, 2001). However, others failed to observe E₂-mediated ERK activation in MCF7 cells (Dupont *et al.*, 2000; Lobenhofer *et al.*, 2000; Caristi *et al.*, 2001), and E₂-induced ERK activity may not be a result of activation of the Ras/Raf signal transduction pathway but may follow rapid increase in intracellular Ca²⁺ (Improta-Brears *et al.*, 1999). Furthermore, we had previously demonstrated that increased cyclin D1 synthesis in response to estrogens is partly blocked by the MEK1 inhibitor PD98059 and that dominant negative ERK2 partially inhibited the induction of cell cycle transit by estrogens (Ahamed *et al.*, 2001). Therefore, we measured a detailed time course of ERK activation by E₂. We found that E₂ rapidly activated ERK2 with maximum activation of fivefold observed within 15 min (Figure 7A) and significant ERK2 activation was sustained for at least 120 min. Enhancement of ERK activity by E₂ was partly blocked not only by pretreatment with the PI-3K inhibitor LY294002 but also by the specific estrogen antagonist ICI182,780 (Figure 7B). Given the above-mentioned results and recent work indicating that there is cross talk between the PI-3K and ERK pathways (Hawes *et al.*, 1996; Wennstrom and Downward, 1999), we explored this interaction further by overexpression of adenoviral PTEN. Whereas E₂ or IGF-1 activated ERKs in control Ad virus-infected cells, PTEN overexpression (~3 $\times$ over the basal level; Figure 7C, 3) partly blocked ERK activation observed at 15 min of treatment with these two agonists (Figure 7C, 1). In confirmation of these data by using phospho-specific ERK antibodies, PTEN also partly inhibited ERK2 activity measured by MBP phosphorylation in an in vitro kinase assay (Figure 7C, 2). In extracts from similarly treated cells, BAD phosphorylation in vitro by Akt and p90^{RSK1} was also selectively abrogated by PTEN overexpression (Figure 7D), demonstrating that PTEN overexpression inhibited Akt and p90^{RSK1} activation. These results suggest that the PI-3K pathway may regulate the ERK response in addition to the Akt response in MCF7 cells and provide evidence for cross talk between these signal pathways in response to E₂ or IGF-1 under our experimental conditions.

DISCUSSION

Regulation of Apoptosis by E₂

E₂ is known to support cell survival or induce cell death/apoptosis depending on the cell context (Choi *et al.*, 2001; Okasha *et al.*, 2001). Most of E₂'s actions on cell survival or death, as for many other steroid hormone actions, have been attributed to gene regulation. Emerging evidence for nongenomic actions of E₂ (reviewed in Kelly and Levin, 2001) prompted us to examine the signaling mechanisms of E₂ with respect to regulation of cell survival in MCF7 breast cancer cells. We had previously shown that estrogens were able to decrease apoptosis induced by mitogen withdrawal in MCF7 cells (Ahamed *et al.*, 2001). Our present study shows that E₂ significantly reduces apoptosis induced by TNF- α , H₂O₂, and serum withdrawal by using signal transduction pathways that lead to phosphorylation and inactivation of proapoptotic protein BAD. In Figure 8 we summarize our results and present a

Figure 6. 17- β -Estradiol activates the PI-3K/Akt pathway. Serum-deprived MCF7 cells were treated with E₂ (10 nM) or IGF-1 (10 ng/ml) for indicated times. Immunoprecipitates of PI-3K (A), GSK3 β (C), and p90^{RSK1} (E) were subjected to in vitro kinase assay with respective substrates in the presence of [γ -³²P]ATP. Reactions were resolved by SDS-PAGE, and autoradiograms were obtained. Fold activation/inhibition compared with time 0 was determined by densitometry. Representative autoradiograms are shown along with graphs depicting mean values ($\pm$ SD) of three or more independent experiments. Protein level of p90^{RSK1} at these time points is shown below E. (B) Graph represents results (mean $\pm$ SD) of three independent in vitro kinase assays for Akt with BAD as substrate (**p < 0.005 or *p < 0.05 compared with time 0). (D) Immunoprecipitates of p90^{RSK1} from cells treated as in A were subjected to in vitro kinase assay with agarose conjugated BAD. BAD was collected, resolved on SDS-PAGE, and Western blotted with anti-phospho S112 BAD antibody, and fold stimulation was determined by densitometry. The experiment was repeated two times with comparable results.

Figure 7. 17- β -Estradiol induces activity of ERKs and p90^{RSK1}. (A) Serum-starved MCF7 cells were treated with E₂ (10 nM) for indicated times. ERK2 immunoprecipitates were subjected to in vitro kinase assay with MBP as a substrate. Band intensities were quantified by densitometry. Graph presents data from three experiments (* $p < 0.05$ and ** $p < 0.005$ compared with time 0). (B) MCF7 cells were treated with E₂ (10 nM) with or without pretreatment of LY294002 (20 μ M) or ICI182,780 (1 μ M) for 1 h. Cell lysates were assayed by Western blot by using phospho-ERK and ERK2 antibodies. (C) Adenoviral PTEN and Lac-Z (control)-infected cells were serum deprived and treated with IGF-1 (10 ng/ml) or E₂ (10 nM) for 15 min. Panel 1, Western blot analyses of cell lysates with phospho-ERK and ERK2 antibodies. Panel 2, ERK2 immunoprecipitates from the above-mentioned lysates were subjected to in vitro kinase assay with MBP in the presence of [γ -³²P]ATP, and band intensities are presented in the graph. Data shown are representative of three independent experiments. Panel 3 shows the overexpression of PTEN by the viral vector. (D) Cells were treated as in C. Akt and p90^{RSK1} immunoprecipitates were subjected to in vitro kinase assays with BAD as a substrate in the presence of [γ -³²P]ATP. Band intensities of two experiments are shown in the graphs.

Figure 8. Schematic diagram showing the proposed survival mechanism induced by 17- β -estradiol. Estradiol activates ERK/p90^{RSK1} and PI-3K/Akt pathways possibly through Ras-dependent mechanisms. On activation of these signal pathways, proapoptotic BAD is phosphorylated at S112 and S136. Phosphorylated BAD is sequestered by 14-3-3 (and possibly by other molecules), thus preventing its action on the mitochondrial membrane. This mechanism ensures cell survival and may constitute the antiapoptotic effects observed when the apoptotic stimuli are present simultaneously with E₂. E₂ could not exert this protective effect when phosphorylation sites in BAD are mutated to unphosphorylatable residues. E₂-mediated signaling pathway is indicated by black arrows. Apoptosis-inducing agents change mitochondrial membrane integrity and facilitate the release of substances such as cytochrome *c* into the cytoplasm. These in turn mediate a cascade of reactions, eventually leading to apoptosis that can be readily identified by characteristic features pointed out in the figure. Inhibition of BAD protein level by antisense mRNA could drastically reduce the amount of apoptosis observed with apoptotic agents, providing evidence for the critical involvement of BAD in this scenario. Signal pathways mediated by apoptotic stimuli are indicated by white arrows. Dominant negative Ras (RasN17) dramatically increases apoptosis even under exponential growth conditions and blocked the protective effects of E₂, suggesting an essential cell survival role for Ras.

model on how E₂ is able to promote cell survival and abrogate apoptosis in MCF7 cells. However, E₂ is unable to counteract the apoptosis-inducing effects of other agents such as paclitaxel (our unpublished data); this observation is in contrast to recently published work (Razandi *et al.*, 2002).

Role of BAD in Apoptosis and Its Regulation by E₂

E₂ effects on BCL2 family members have been described previously: in cells where survival is promoted, E₂ increased mRNA (Perillo *et al.*, 2000) and protein levels of antiapoptotic BCL2 (Dong *et al.*, 1999; Choi *et al.*, 2001) or down-regulated proapoptotic Bak protein (Leung *et al.*, 1998). In

contrast to regulation of BCL2 and Bak at the transcriptional level, we find that E₂ rapidly induces phosphorylation of BAD at two serine residues (112 and 136) without altering BAD protein levels (Figure 3B), suggestive of activation of nongenomic signal pathways. Given the fact that proapoptotic effects of BAD are decreased by phosphorylation and consequent sequestration by 14-3-3 proteins (Downward, 1999), we sought to determine whether E₂-induced phosphorylation of BAD is important for the antiapoptotic functions of E₂.

BAD complexes with BCL2 and BCLxL, and leads to loss of mitochondrial integrity (Hirai and Wang, 2001); therefore, release of cytochrome *c* into the cytoplasm is expected after BAD "activation." The apoptotic stimuli increased the cytochrome *c* levels in the cytoplasm, and cotreatment with E₂ reduced this effect concomitant with improved cell survival (Figure 1D). This suggests the restoration of mitochondrial integrity at least to some degree in E₂-treated cells and may account for antiapoptotic effects of E₂. In cells treated with TNF- α and H₂O₂, the extent of S112/136 BAD phosphorylation was lower than that observed in control cultures (Figure 3C), implicating the activation of BAD by these apoptotic stimuli. Estrogen treatment in this context restored phosphorylation to control levels. These experiments, along with those demonstrating that E₂ prevents apoptosis in cells overexpressing wild-type but not the double phosphorylation site mutant (S112A/S136A) of BAD (Figure 4, E–F), suggest that BAD phosphorylation has a central role in apoptosis of MCF7 cells. Remarkably, the overexpression of BAD (S112A/S136A) did not cause apoptosis by itself, suggesting that in addition to phospho-serine-mediated cytosolic 14-3-3 binding there may be other mechanisms to sequester BAD away from mitochondria. These mechanisms effectively inactivating BAD are counteracted by apoptotic stimuli such as TNF- α . Binding of BAD to other cellular proteins such as glucokinase has been demonstrated in the rat liver (Danial *et al.*, 2003).

To confirm the importance of BAD in MCF7 cells, we used antisense oligonucleotides to suppress BAD protein translation (Figure 4A) and analyzed the effects of selected agents on MCF7 cell survival. Diminution of BAD in MCF7 cells significantly reduced the extent of apoptosis induced by TNF- α (Figure 4, B–D). This observation is in accordance with previously published studies where overexpression of BAD was shown to enhance cell death (Otilie *et al.*, 1997; Virdee *et al.*, 2000). The fact that antisense BAD blocked ability of TNF- α to induce apoptosis (Figure 4, C and D) suggests that BAD phosphorylation/inactivation has a central role in apoptosis of MCF7 cells in response to stimuli such as TNF- α .

Agents such as TNF- α and H₂O₂ have been shown to have mixed effects on BAD protein levels and phosphorylation. TNF- α increased phosphorylation of BAD through PI-3K pathway in neutrophils (Cowburn *et al.*, 2002), and in HeLa cells (Pastorino *et al.*, 1999) where cell survival rather than the cell death was promoted. TNF- α also induced the production of more potent truncated form of BAD in a caspase 3-dependent manner, leading to apoptosis in Jurkat cells (Condorelli *et al.*, 2001). Similarly, H₂O₂ but not superoxide anion (O₂⁻) has been shown to increase BAD protein levels in cardiomyocytes, leading to apoptosis (von Harsdorf *et al.*, 1999). Serum starvation may modulate activity of BAD and other apoptosis regulators, and such effects may be reversed by addition of growth factors or by manipulating signal transduction pathways (Yano *et al.*, 1998; Bai *et al.*, 1999). Recent evidence from BAD knockout mice suggests that BAD may have an important proapoptotic role in certain cell

types such as the mammary tissue. Importance of BAD was especially underscored in apoptosis that occurs in the absence of growth factors (Ranger *et al.*, 2003) much like the importance of BAD inactivation in the response to E₂, which is a well-known growth factor to breast cancer cells. However, it is also evident that BAD concentration or phosphorylation status could not be used to define survival or apoptosis in other cells such as PC12 cells (Maroto and Perez-Polo, 1997).

Estradiol Signals through Two Pathways in Its Antiapoptotic Action

Roles of Ras/PI-3K/Akt and Ras/ERK/p90^{RSK} Pathways in BAD Phosphorylation. The ability of DN Akt or PTEN to reverse the protective effects of E₂ (Figure 2) suggests an essential role for signal transduction through PI-3K/Akt in E₂ action in protecting cells from apoptosis. Akt is a known oncogene and is overexpressed in breast cancer cell lines compared with untransformed breast epithelial cells, as well as in breast cancer specimens where the majority of cells are ER positive (Sun *et al.*, 2001). Deranged PI-3K/Akt signaling due to PTEN deficiency or germline mutation has been proposed as a frequent cause for breast cancer (Mills *et al.*, 2001; Petrocelli and Slingerland, 2001). Given the importance of PI-3K/Akt pathway to antiapoptotic action of E₂, we examined aspects of this pathway, which might influence cell survival. Our results clearly indicate that PI-3K/Akt has a critical role in E₂ action as a survival agent (Figure 1A, 2). While this work was in progress, Fernando and Wimalasena (2000, 2002), Castoria *et al.* (2001), and Stoica *et al.* (2003a) demonstrated that E₂ rapidly activates PI-3K/Akt in MCF7 cells, but others were unable to observe such activities of E₂ (Ahmad *et al.*, 1999; Dupont *et al.*, 2000). However, in contrast to Castoria *et al.* (2001), we found a rapid and sustained activation of Akt in response to E₂ (Figure 6B). The reasons for these disparities, including conflicting results from one group (Ahmad *et al.*, 1999; Stoica *et al.*, 2003a) are not clear but could relate to subtle culture conditions, treatment periods, or cell line variability. Prolonged activity of Akt is required to maintain BAD phosphorylation at S136 (Figure 3B; our unpublished data) as well as to maintain GSK3 β in the inactive state (Figure 6C; our unpublished data). Active GSK3 β may be required to stabilize cyclin D1 (Diehl *et al.*, 1998) and increased cyclin D1 protein is a characteristic response to E₂ in MCF7 and other estrogen-responsive cells (Foster *et al.*, 2001, and references therein). E₂ signaling also has been shown to be closely associated with the PI-3K/Akt pathway in vascular endothelial cells (Hisamoto *et al.*, 2001), and ER α is phosphorylated by activation of the PI-3K/Akt pathway in MCF7 cells in the absence of E₂ (Campbell *et al.*, 2001; Stoica *et al.*, 2003b).

In addition to Akt-mediated signaling, our data demonstrate that E₂ also may signal through Ras/ERK/p90^{RSK} pathway because endogenous and exogenous BAD were effectively phosphorylated at S112, a well-known ERK/p90^{RSK} phosphorylation site (Downward, 1999) in E₂-treated cells (Figure 3, A and B). Our studies with activating and inactivating Ras mutants provides strong evidence for this line of argument and show that BAD S112 is a target of Ras-mediated MEK/ERK activity (Figure 5B). We could directly demonstrate that E₂ is able to activate p90^{RSK1} (Figure 6, D and E), a downstream target of ERK1 and 2 (Zhao *et al.*, 1996; Bhatt and Ferrell, 1999). Although the ability of E₂ to activate ERK remains controversial (Lobenhofer *et al.*, 2000; Caristi *et al.*, 2001; Song *et al.*, 2002) due to unknown reasons, ERK activation seems to be important in the apoptotic re-

sponse (Fang *et al.*, 1999; Shimamura *et al.*, 2000), perhaps through the regulation of BAD phosphorylation via p90^{RSK}. The prolonged activation of ERK (Figure 7A) in contrast to transient activation reported by others (Migliaccio *et al.*, 1996) may be important to maintain the phosphorylation and inactivation of BAD, acting in concert with Akt. Prolonged ERK activation is also required for G1/S transition via enhanced cyclin D1 synthesis (Murphy *et al.*, 2002) and p27^{kip1} degradation (Foster, 2003). Together, the data in Figure 5 implicates a critical role for Ras/Raf/ERK in E₂-promoted MCF7 cell survival. It is also possible that BAD can be phosphorylated by kinases other than Akt and p90^{RSK} and that such phosphorylations may enhance or diminish the proapoptotic capacity of BAD (Harada *et al.*, 1999; Lizcano *et al.*, 2000; Donovan *et al.*, 2002; Konishi *et al.*, 2002).

Interaction and Regulation of the Estradiol-induced Signal Pathways. The two signaling pathways that we describe here may function as separate entities or interact to mediate E₂ effects. Previous studies have shown that Ras/ERK and PI-3K/Akt exhibit regulatory cross talk (Wennstrom and Downward, 1999; Shields *et al.*, 2000; York *et al.*, 2000). Regulation of ERK by E₂ through the PI-3K/Akt pathway has not been previously reported to our knowledge. Using both biological and chemical inhibitors, we found that PI-3K may be required not only for E₂ but also for IGF-1-induced ERK activation (Figure 7, B and C) as well as BAD phosphorylation at S112, a likely target of the ERK/p90^{RSK} pathway (Figure 5A). Therefore, it is likely that E₂ regulates ERK activation, in part, through PI-3K pathway, and our data suggest that E₂ regulates a step upstream of PI-3K, possibly Ras function (Rodriguez-Viciana *et al.*, 1994). Overexpression of dominant negative Ras (RasN17) interfered with E₂-induced BAD phosphorylation on both serine residues (Figure 5B) without affecting the basal BAD phosphorylation or protein levels supporting the notion that E₂ regulates PI-3K through Ras (Khwaja *et al.*, 1997). However, we cannot exclude the possibility that E₂ may regulate another kinase, which can phosphorylate BAD on S136. We previously demonstrated a role for Ras in estrogen-induced cell cycle progression (Ahamed *et al.*, 2001; Zhu *et al.*, 2003) and recently showed that the Ras/Raf/ERK pathway has a critical role in E₂ induced p27^{kip1} degradation and relocalization in MCF7 cells (Foster, 2003). Others (Migliaccio *et al.*, 1996; Filardo *et al.*, 2002) have reported rapid activation of Ras by E₂. The ability of RasN17 to induce apoptosis in proliferating cells (Figure 5C) suggests that Ras plays a critical role in preventing apoptosis in MCF7 cells as observed in other cells (Wolfman *et al.*, 2002). Because 3'4'5' or 3'4'PI phosphates regulate a variety of signal pathways such as Akt and PDK that could lead to modulation of Ras/Raf/ERK pathway (Rameh and Cantley, 1999), nongenomic signaling by E₂ may be very complex (Levin, 2003). Furthermore, activation of PI-3K by E₂ through a direct interaction of the p85 subunit with ERα was reported in human vascular endothelial cells (Simoncini *et al.*, 2000) and MCF7 cells (Sun *et al.*, 2001). Clearly, further studies are needed to delineate signaling mechanisms and their interactions in the perspective of estradiol's antiapoptotic and cell cycle effects.

Interestingly, many of the responses to E₂ observed in this study also were elicited by IGF-1, as has been demonstrated in the context of MCF7 cell proliferation (Stewart *et al.*, 1990; Dupont *et al.*, 2000). E₂ may indirectly activate Akt genomic actions (increased IGF-1 receptor expression, IGF-1 synthesis/secretion) (Ahmad *et al.*, 1999; Lee *et al.*, 1999); however, the responses we have described here are elicited by E₂

within a 2- to 15-min time frame, which is not tenable with genomic actions of E₂. On the other hand, we cannot formally exclude the possibility that E₂ may activate immediate release of IGF-1 (or other growth factors) from an inactive storage form(s), as recently documented (Filardo *et al.*, 2000) for epidermal growth factor. The nongenomic signaling of E₂ is postulated to be mediated by membrane-associated ER (Kelly and Levin, 2001), and this hypothesis is further supported by the recent demonstrations of membrane located ERα in MCF7 and other cell types (Marquez and Pietras, 2001; Powell *et al.*, 2001). According to these studies, the fraction of plasma membrane-bound ERα ranged from 3 to 20% of the total in MCF7 cells. Agreeing with this notion, our data show that E₂ induced rapid ERK phosphorylation, and the protective effects against apoptotic agents was substantially blocked by the antiestrogen ICI182,780 (Figures 1B and 7B).

SUMMARY

This study demonstrates that E₂ as well as IGF-1 inhibit apoptosis in MCF7 cells by enhanced phosphorylation of BAD and indicates that BAD inactivation results from activation of Ras/ERK/p90^{RSK} and Ras/PI-3K/Akt pathways by E₂. Our results also indicate that BAD plays an essential role in apoptosis induced by H₂O₂, TNF-α, and serum withdrawal and also demonstrate that even the double phosphorylation site mutant of BAD (S112/136A) is inactive as an inducer of apoptosis in the absence of TNF-α. Our results suggest that the ability of E₂ to prevent apoptosis in MCF7 cells requires BAD inactivation, which occurs in response to Ras activation by E₂. Thus, we have demonstrated a novel nongenomic pathway by which E₂ promotes survival of breast cancer cells in culture. Furthermore, this is the first report to our knowledge demonstrating the role of the Ras/PI-3K/Akt/BAD and Ras/Raf/ERK/p90^{RSK}/BAD pathways as potential nongenomic mediators of the antiapoptotic actions of E₂ or any other steroid hormone. The crucial contributions of aberrant Ras as well as those of Akt function to human cancer development is widely accepted (Datta *et al.*, 1999; Shields *et al.*, 2000), and inactive mutations or down-regulation of tumor suppressor PTEN have been linked to accelerated tumor growth in breast and other cancers (Maehama and Dixon, 1999). Therefore, our study provides a mechanism whereby the functions of these oncogenes and tumor suppressors may converge and dictate the fate of the breast cancer cell.

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