Recent investigations suggest that functions of the proapoptotic BCL2 family members, including BAD, are not limited to regulation of apoptosis. Here we demonstrate that BAD inhibits G1 to S phase transition in MCF7 breast cancer cells independent of apoptosis. BAD overexpression inhibited G1 transit and cell growth as well as cyclin D1 expression. Inhibition of cyclin D1 expression was mediated through inhibition of transcription activated by AP1. Chromatin immunoprecipitation assays indicated that BAD is localized at the 12-O-tetradecanoylphorbol-13-acetate-response element (TRE) and cAMP-response element (CRE) in the cyclin D1 promoter. This was shown to reflect direct binding interactions of BAD with c-Jun, and this interaction inhibited the activity of AP1 complexes at TRE. BAD did not interact with phosphorylated forms of c-Jun. Our data suggest that inhibitory TRE/CRE-c-Jun-BAD complexes are present at the cyclin D1 promoter in quiescent cells. Estrogen stimulation displaced BAD from TRE/CRE elements in MCF7 cells, whereas BAD overexpression inhibited estrogen-induced cyclin D1 synthesis and cell proliferation. Inhibition of endogenous BAD in MCF7 cells markedly increased the proliferative fraction and DNA synthesis, activated Cdk5, and increased cyclin D1 protein levels. This action of BAD required serine residues Ser75 and Ser99. Both phosphorylated and unphosphorylated forms of BAD localized to the nuclei of human breast epithelial cells. Thus, we demonstrate a novel role for BAD in cell cycle regulation dependent upon its phosphorylation state and independent of the BAD/BCL2 interaction and apoptosis.

Recent studies on BCL2 family proteins suggest that members of the proapoptotic arm have functions distinct from apoptosis induction. BAD has been shown to have a role in the DNA damage response (1), and BAX and BID take part in homologous recombination (2). A prosurvival role for BAD was demonstrated in murine neuronal cells (2), and metabolic functions of BAD were described in mice (3). Growth regulation by BCL2 family proteins has been previously reported, although the mechanisms are poorly defined (4–6). BAD is devoid of the membrane-anchoring hydrophobic region present in most other BCL2 proteins and it resides primarily in the cytoplasm. Under apoptotic conditions, BAD is found in the mitochondria, binding to BCL2 and BCLxL (7, 8). Cell survival signals induce inhibitory phosphorylation of BAD on Ser75 and Ser99 (murine Ser112 and Ser136, respectively) and promote its sequestration by 14-3-3 proteins. This mechanism obliterates the inhibitory effects of BAD on pro-survival BCLxL/BCL2 (9).

We previously showed in MCF7 breast cancer epithelial cells that estradiol inhibits apoptosis by inducing phosphorylation of BAD (10), and a role for BAD in regulating apoptosis in murine breast epithelial cells has been described in vivo (9). In our studies, we consistently observed that mere overexpression of BAD in MCF7 cells did not cause apoptosis; rather there was an enhanced response once the cells were exposed to an apoptotic stimulus. This suggests levels of regulation between cytoplasmic BAD and its binding and inhibition of BCL2/BCLxL in mitochondria. Similar to our work in MCF7 cells, studies in BAD knock-out mice demonstrated the importance of BAD in survival promotion by growth factors (9). Furthermore, cleavage of long form BAD to a short form was required for its apoptotic role (3). These studies taken together indicate that BAD, by itself, does not necessarily cause apoptosis.

In our previous study (10) and in unpublished work, we observed that some portion of BAD localizes in the nucleus and that BAD overexpression led to decreased cell proliferation. In this study, we have studied in detail the cell cycle regulation of MCF7 cells by BAD. This cell line has been utilized as the premier model of hormone-sensitive (estrogen receptor-positive) breast cancer by many laboratories, including our own. In MCF7 cells, cyclin D1 has a rate-limiting role in G1-S phase transition (11–13), and both transgenic mouse models and clin-

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**REFERENCE**

Romaine Fernando, James S. Foster, Amber Bible, Anders Ström, Richard G. Pestell, Mahadev Rao, Arnold Saxton, Seung Joon Baek, Kiyoshi Yamaguchi, Robert Donnell, Maria Cekanova, and Jay Wimalasena

From the 4Graduate School of Medicine, University of Tennessee Medical Center, Knoxville, Tennessee 37920, the 6Department of Biosciences, Karolinska Institute, Novum, S-14157, Sweden, the 6Departments of Cancer Biology and Medical Oncology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, the 6Department of Oncology, NCI, National Institutes of Health, Washington, D. C. 20057, the **Department of Animal Science, University of Tennessee, Knoxville, Tennessee 37996, and the ‡**Department of Pathobiology, University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee 37996
ical studies indicate a pivotal role for cyclin D1 in normal and malignant cell growth, particularly in breast cancer (14–16). Our data strongly suggest that cyclin D1 is an important target of the inhibitory cell cycle effects of BAD. We also show that the dual phosphorylation site mutant of BAD (dmBAD),5 with mutations at S75A and S99A, is ineffective in many aspects of cell cycle regulation, suggesting a requirement for phosphorylation at these residues, which are also targets of prosurvival mechanisms. Accordingly, the BH3 domain deletion mutant and the S91A mutant of BAD, both of which carry intact Ser75 and Ser99 residues, exerted cell cycle-inhibitory effects similar to that of wild type BAD.

Collectively, our data suggest that BAD may act to prevent tumor growth both via proapoptotic mechanisms and by antiproliferative actions of its phosphorylated, apoptotically “inactive” form. Given the importance of cyclin D1 in proliferation of many tumor cells, regulation of cyclin D1 levels by BAD may have a crucial role in cell cycle control. Selectively increasing BAD expression in breast cancer cells may lead to decreased tumor growth in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—MCF7 cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 5% fetal bovine serum, penicillin G, and streptomycin at 37 °C in 5% CO2. Cells were transfected using Lipofectamine Plus™ reagent (Invitrogen). FLAG-BAD (human) vectors were provided by Dr. H. G. Wang (17), and the BH3 domain-deleted mutant was a gift from Dr. A. Kelekar (8). The luciferase vector transfection, pEGFPN1 (Clontech) was measured using a scintillation counter. Intensity of the band denotes the amount of nuclear extract (prepared as described in the supplemental materials), radiolabeled probe, and gel shift binding buffer with the relevant volume of nuclease-free water. At the end of the incubation period, reactions were resolved on denaturing acrylamide gels and dried, and autoradiograms were obtained.

Reporter Assay—AP1/TRE-luciferase and cyclin D1-luciferase constructs (19) were transfected into MCF7 and T47D cells. Cells were rinsed with phosphate-buffered saline and lysed with 1× reporter lysis buffer (catalog number E3971; Promega) on ice for 10 min. Supernatant was collected after brief centrifugation (12,000 × g for 2 min at 4 °C). One hundred micrograms of cell lysate was assayed with 100 µl of luciferase assay substrate (catalog number E4030; Promega), and light emission was measured using a scintillation counter. To equalize levels of luciferase vector transfection, pEGFPN1 (Clontech) was co-transfected, and EGFP levels were analyzed by Western blotting.

Metabolic Labeling with [35S]Methionine—Cells were cysteine/methionine-deprived for 1 h (methionine-free Dulbecco’s modified Eagle’s medium/F-12) and labeled with 75 µCi/ml Tran35S-label (MP Biomedical) in the same medium as indicated. Labeling medium was removed, and the cells were washed twice with cold phosphate-buffered saline. Cells were lysed, and proteins were immunoprecipitated from whole cell extracts, resolved using 12% SDS-PAGE gels, and visualized by autoradiography. Intensity of the band denotes the amount of [35S]methionine incorporated within the labeling period (20).

Reverse Transcription-PCR and Northern Blotting—Total cellular RNA was extracted from MCF7/BAD cells (Tet+ or Tet−) using Perfect RNA (Eppendorf). One microgram of total RNA was reverse transcribed according to the manufacturer’s protocol (iScript; Bio-Rad). The synthesized cDNA was then added to a 25-µl PCR mixture (REDTag ReadyMix PCR mixture; Sigma) with each set of gene-specific primers: cyclin D1, forward (5’-atgaaaccagcctgtctggt-3’) and reverse (5’-tca-gactgctcagctcgcagGtG-3’); gliceraldehyde-3-phosphate dehydrogenase, forward (5’-ggctgctttaactctggt-3’) and reverse (5’-ttggagtttttagccag-3’). The thermal cycling conditions were as follows. Initial denaturation at 94 °C for 3 min, followed by 25–30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The final PCR products were electrophoresed on 1% agarose gels and photographed under UV light. Northern blotting for cyclin D1 was carried out as in Ref. 21.

Inhibition of BAD Expression—siRNA for human BAD (5’-GAAGGGACUUCCUCGCCCGTT-3’, 5’-CGGGCGAGGA- AGUCCCUUTC-3’) (catalog number 6471; Cell Signal Technology) and control siRNA (5’-CGUACCGGAAUACUCUC- GATT-3’, 5’-UCGAAGAUUCCCGGUAAGTT) (catalog number 6201) were transfected at a final concentration of 33 nM using RNAifect transfection reagent according to the manufacturer’s directions (Qiagen).

Electrophoretic Mobility Shift Assay (EMSA)—The TRE and control oligonucleotides were end-labeled with [γ-32P]ATP and 4% nondenaturing acrylamide gels were prepared according to the manufacturer’s protocol (catalog number E3300; Promega). DNA binding reactions were carried out using 50 µg of nuclear extract (prepared as described in the supplemental materials), radiolabeled probe, and gel shift binding buffer with the relevant volume of nuclease-free water. At the end of the incubation period, reactions were resolved on nondenaturing acrylamide gels and dried, and autoradiograms were obtained.

Establishment of MCF7/BAD Cells—MCF7 cells stably transfected with tetracycline (Tet)-regulated BAD expression plasmid were generated in two steps. The cells were first transfected with pTet-tTAK (Invitrogen), modified to contain the puromycin resistance gene, using Lipofectin (Invitrogen) according to the manufacturer’s instructions. Selection was performed with 0.5 µg/ml puromycin (Sigma) in the presence of 1 µg/ml Tet. A clone showing high Tet-controlled transactivator tTA induction upon Tet withdrawal and low basal activity was selected by the relevant volume of nuclease-free water. At the end of the incubation period, reactions were resolved on nondenaturing acrylamide gels and dried, and autoradiograms were obtained.

Reverse Transcription-PCR and Northern Blotting—Total cellular RNA was extracted from MCF7/BAD cells (Tet+ or Tet−) using Perfect RNA (Eppendorf). One microgram of total RNA was reverse transcribed according to the manufacturer’s protocol (iScript; Bio-Rad). The synthesized cDNA was then added to a 25-µl PCR mixture (REDTag ReadyMix PCR mixture; Sigma) with each set of gene-specific primers: cyclin D1, forward (5’-atgaaaccagcctgtctggt-3’) and reverse (5’-tagctgcacgctgcagGtG-3’); gliceraldehyde-3-phosphate dehydrogenase, forward (5’-ggctgctttaactctggt-3’) and reverse (5’-ttggagtttttagccag-3’). The thermal cycling conditions were as follows. Initial denaturation at 94 °C for 3 min, followed by 25–30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min. The final PCR products were electrophoresed on 1% agarose gels and photographed under UV light. Northern blotting for cyclin D1 was carried out as in Ref. 21.

5 The abbreviations used are: dmBAD, dual phosphorylation site mutant of BAD; Tet, tetracycline; EMSA, electrophoretic mobility shift assay; ChiP, chromatin immunoprecipitation assay; RNAi, RNA interference; wtBAD, wild type BAD; E2, estradiol; CREB, cAMP-response element-binding protein.
Cell Cycle Functions of BAD

Chromatin Immunoprecipitation Assay (ChIP)—ChIP analysis was performed following a protocol (Upstate Biotechnology, Inc.) with modifications. MCF7 cells were grown in Dulbecco’s modified Eagle’s medium with 10% charcoal-stripped dextran serum for 3 days. Upon estradiol (100 nm) stimulation for 45 min, the cells were cross-linked using 1.1% formaldehyde buffer containing 100 mm sodium chloride, 1 mm EDTA-Na (pH 8.0), 0.5 mm EGTA-Na, Tris-HCl (pH 8.0) for 10 min at 37 °C. The cells were washed three times with ice-cold phosphate-buffered saline containing 10 mm dithiothreitol and protease inhibitors and lysed by warm 1% SDS lysis buffer and incubated for 10 min on ice. Cell lysates were sonicated to shear DNA to lengths between 200 and 1000 bp, and these samples were diluted 10-fold in ChIP dilution buffer. Lysates were precleared with 60 μl of salmon sperm DNA/Protein A-agarose 50% slurry (Upstate Biologicals) for 2 h at 4 °C with agitation and chromatin immunoprecipitated overnight at 4 °C using 4 μg of anti-BAD (Upstate Biologicals). Rabbit IgG (negative control) was incubated for 1 h at 4 °C. salmon sperm DNA/Protein A-agarose slurry (60 μl) was added for 2 h at 4 °C with rotation to collect the antibody-protein complex and washed extensively following the manufacturer’s protocol. Input and immunoprecipitated chromatin were incubated at 65 °C overnight to reverse cross-links. After proteinase K digestion for 1 h, DNA was extracted using Qiagen spin column kit. Precipitated DNA was analyzed by PCR for human cyclin D1 promoter using following primers: AP1, sense (5′-CTGCCCTTCTACTTTGAGC-3′) and antisense (5′-TGAAAGGAGCTCTACCCCCC-3′); cAMP response element (CRE), sense (5′-GCCCCCCTC-CGGTCCCCATT-3′) and antisense (5′-TGGGGCTCTTCTCTGGGCAGC-3′).

Statistical Methods—Analyses were done using GraphPad Prism version 4. Data were analyzed with two-way analysis of variance blocked by experiment, and treatment means were compared within times using pairwise contrasts at the 5% significance level.

Other Methods—DNA synthesis was assayed by bromodeoxyuridine incorporation according to the manufacturer’s instructions (catalog number 559619; BD Pharmacien). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays, DNA fragmentation enzyme-linked immunosorbent assay, microscopic analysis of cells, Western blotting, subcellular fractionation, flow cytometry analysis, and in vitro kinase assays were performed as described previously (10, 20), and details are provided in the supplement.

RESULTS

BAD Inhibits Cell Growth and S Phase Entry in MCF7—We previously demonstrated that BAD is one target of estrogen-induced cell survival in MCF7 cells and that transient overexpression of BAD does not result in apoptosis in these cells under normal growth conditions (10). In our subsequent studies, a noticeably slow growth rate was observed in MCF7 cells overexpressing BAD. MCF7 cells have been used by numerous investigators as a tissue culture model to identify molecules regulating cell growth and apoptosis in hormone-responsive breast cancer. In the present study, we investigated in detail the growth characteristics of MCF7 cells with varying levels of BAD expression. We generated a cell line (MCF7/BAD) that conditionally expresses BAD under a Tet-regulated promoter to complement the results obtained by transient overexpression. Both methods yielded similar results. Withdrawal of Tet for 48–72 h elevated the BAD protein level without eliciting apoptosis, as measured by cytoplasmic cytochrome C content and DNA fragmentation enzyme-linked immunosorbent assay for up to 96 h (Fig. 1, A and B, respectively). These cells accumulated at G1 phase of the cell cycle, reflective of inhibited S phase entry (Fig. 1C). Note that BAD did not change the sub-G1 fraction, confirming the absence of apoptotic effects. Cell growth in asynchronized cultures was assayed using MTT uptake (Fig. 1D). BAD overexpression for 120 h suppressed cell growth by 50–60% relative to control cells. Note that in BAD-overexpressing (Tet−) cells, there was no net growth between 72 and 120 h, in contrast to uninduced (Tet+) cells, where continued proliferation was observed (Fig. 1D and supplemental Fig. 1A). Growth suppression elicited by elevated BAD (Tet−48 h) could be significantly (p = 0.0002) reversed by ending BAD overexpression through the readdition of Tet to the culture medium. Induced cultures (Tet+) stop proliferating by 96 h, whereas cultures where Tet was readded (Tet+/ Tet −) continue to proliferate through 120 h. (supplemental Fig. 1A shows statistical analyses of data used for the graph). Clearly, once BAD overexpression has culminated, these cells are able to proliferate. This supports our previous observations that transient overexpression of BAD, by itself, does not induce apoptosis in MCF7 cells (10). Collectively, the data indicate that BAD may regulate cycle progression in MCF7 cells. Importantly, this effect of BAD is independent of apoptosis and is reversible.

BAD Alters Expression and Activity of Cell Cycle Proteins, Including Cyclin D1—To further understand the cell cycle effects of BAD, the expression and activities of critical cell cycle molecules were assayed in conjunction with enforced BAD overexpression or inhibition of endogenous BAD in MCF7 cells. Decreased cyclin D1 protein levels and hypophosphorylation of retinoblastoma protein (pRB) at serine 780 were consistently detected in cells expressing increased levels of BAD (Fig. 2A, time points in duplicate and histogram reflecting results from several experiments). Initial (24–48 h) reduction in pRB phosphorylation is possibly due to decreased cyclin D1-Cdk4 activity, since pRB phosphorylation at serine 780 is specific for Cdk4 (22). The sustained decrease in pRB phosphorylation may also reflect decreased Cdk2- and cyclin B-associated Cdk1 activity (supplemental Fig. 2A). We should note that BAD induction does not completely abolish Cdk activities at 48–72 h, in particular that of Cdk2 (supplemental Fig. 2A). Residual Cdk activities may explain the increase in viable cells between 48 and 72 h (Tet− in Fig. 1D). Cyclin D1 governs cell cycle entry and is rate-limiting for G1-S transition in MCF7 cells (11–13). We hypothesized that the inhibition of G1-S transition by elevated BAD expression (Fig. 1C) might result at least partly from the inhibition of cyclin D1 expression. In BAD-overexpressing cells, reduction in cyclin D1 protein between 48 and 72 h is accompanied by a clear reduction in cyclin D1 mRNA expression (Fig. 2B). Regulation of cyclin D1 expression
BAD Regulates the Cyclin D1 Promoter—Given the results in Figs. 2 and 3, we suspected that BAD may regulate cyclin D1 transcription. Experiments with human cyclin D1 promoter-luciferase constructs demonstrated that BAD overexpression strongly repressed transcription from the cyclin D1 promoter in proliferating cells (Fig. 4A). Expression of dmBAD, on the other hand, did not influence cyclin D1 promoter activities. In G1-arrested cells, the cyclin D1 promoter activity was very low and not further affected by the presence of BAD. The inability of dmBAD to inhibit either cyclin D1 promoter activity or the synthesis of cyclin D1 protein suggests that the cell cycle-regulatory effect of BAD depends on phosphorylation of Ser\(^{25}\) and Ser\(^{29}\) residues.

AP1-responsive sites in the cyclin D1 promoter, such as AP1/TRE and CRE are important for activation of this promoter in estrogen receptor-α-positive cells (24, 25). Cyclin D1 promoter constructs encoding mutant forms of either TRE or CRE, linked to the luciferase gene, were transfected as before. Co-transfection of BAD failed to suppress cyclin D1 promoter when either of these sites are singly mutated (Fig. 4B). The activity of the TRE/CRE double mutation in response to estrogen and other mitogens is primarily transcripational (11), although translational regulation has also been described (23). Measurements of de novo synthesis of cyclin D1 by metabolic labeling in asynchronously growing MCF7/BAD cells indicated that cyclin D1 protein synthesis was significantly lower than in control cells (Fig. 2C), whereas dmBAD had no effect on cyclin D1 synthesis (supplemental Fig. 2C). These results suggest that a critical effect of BAD in regulating S phase entry is the inhibition of cyclin D1 synthesis contributing to G1 blockade.

BAD RNA Interference (RNAi) Enhances Cell Cycle Transit and Cyclin D1 Expression—Inhibition of endogenous BAD expression with specific siRNA (but not control siRNA) treatment increased DNA synthesis (Fig. 3A) and activated Cdk2 and Cdk1 (supplemental Fig. 2B). Inhibition of BAD expression also increased levels of cyclin D1 (Fig. 3B and C) significantly. Inhibition of endogenous BAD did not, however, result in any apparent change in expression of other BCL2 family proteins (Fig. 3B), demonstrating the specificity of the BAD RNAi. Thus, inhibition of endogenous BAD expression produced effects exactly opposite to that of enforced BAD overexpression in MCF7 cells.

Cell Cycle Functions of BAD

BAD inhibits cell growth and S phase entry in MCF7 cells. A, Tet was withdrawn for the indicated periods of time to overexpress BAD in MCF7/BAD cells. Cytosolic fractions of induced and control (Tet\(^{-}\)) cells were used for detection of cytochrome c (Cy.C). Whole cell lysates were used for detection of BAD by Western blot. Data shown are representative of three independent experiments. B, MCF7/BAD cells were withdrawn from Tet (△) or kept in Tet\(^{+}\) medium (□) for the indicated periods of time. Apoptosis was measured by the DNA fragmentation enzyme-linked immunosorbent assay technique (n = 2 with 4 replicates/treatment). Attached cells and culture supernatants were collected together for the assay in all cases. C, MCF7/BAD cells were withdrawn from Tet for 72 h (BAD) or kept in Tet\(^{+}\) medium (Con). The DNA profile was measured by flow cytometry to determine the cell cycle phase distribution within the cultures. BAD overexpression was verified by the green fluorescence (see “Experimental Procedures”), and DNA was stained with propidium iodide (PI) (n = 3 with duplicates **, p < 0.001, bars ± S.E.). Representative flow data are shown in supplemental Fig. 18. D, MCF7/BAD cells were plated at time 0 h with tetracycline (Tet\(^{-}\)) or without tetracycline (Tet\(^{+}\)). Tet\(^{-}\)/Tet\(^{+}\) denotes cultures in Tet\(^{-}\) medium for 48 h and Tet readdition for specified times (24, 48, and 72 h with total culture ages of 72, 96, and 120 h, respectively). Cell survival was measured by MTT assay in Tet\(^{-}\), Tet\(^{+}\), and Tet\(^{-}\)/Tet\(^{+}\) cultures. The graph represents data from three independent experiments with 3–6 replicates/treatment. **, p = 0.0035 for Tet\(^{-}\) versus Tet\(^{+}\) 120 h. ***, p = 0.0002 for Tet\(^{-}\) versus Tet\(^{-}\)/Tet\(^{+}\) with readdition of Tet for 72 h (culture age 120 h). Bars, ± S.E. Supplemental Fig. 1A shows means and S.E. that were used to obtain the graph and demonstrates that Tet\(^{-}\) cultures were stationary between 72 and 120 h. Statistical methods are described under “Experimental Procedures.”
The inability of the BH3 deletion mutant of BAD to inhibit AP1-dependent luciferase activity suggests that binding to other BCL2 family members is not required for transcriptional effects of this molecule. Similarly, Ser91, a residue phosphorylated by c-Jun N-terminal kinase (26), is not required for repression of AP1-dependent transcriptional activation, whereas Ser75 and Ser99 are absolutely required. These results suggest that BAD is able to inhibit the interaction of AP1 transcription factors with both CRE and TRE motifs. Collectively, the data suggest that BAD action on cyclin D1 expression is at least partly exerted at the transcriptional level and that BAD may repress the function of AP1 transcription factors at their target site, such as TRE and CRE. BAD Modifies AP1 Protein Interaction with Their Targets—Since BAD is devoid of known transcriptional motifs, we postulated that the transcriptional effects on cyclin D1 expression are exerted indirectly via interactions with AP1 proteins. To further evaluate the inhibitory effects of BAD on AP1 transcription factors and DNA interaction, we used a minimal consensus AP1 protein binding DNA sequence (TGAGTCA) in EMSAs. We observed decreased DNA binding upon antibody depletion of BAD from the nuclear extract but not after depletion of BCL2 (Fig. 5A, lane 3 versus lane 4). Similarly, decreased AP1 binding was observed after BAD overexpression (Fig. 5B, lane 3) and after depletion of c-Jun from nuclear extracts (Fig. 5A, lane 5).

FIGURE 2. BAD controls cyclin D1 expression. A, MCF7/BAD cells were withdrawn from Tet for the indicated times, and cell cycle molecules were analyzed by Western blotting (WB). Induced (Tet−) samples are shown from duplicate experiments. The same blots were stripped and reprobed for other indicated molecules. Phosphorylated RB detected with the phospho-Ser330 antibody gave rise to multiple slower migrating bands, as has been shown before (40, 41). Densitometry analysis of cyclin D1 and BAD from three independent Western blots is shown in the graph. **, p < 0.001 for BAD (72 h) versus BAD (control). ***, p < 0.0001 for cyclin D1 (72 h) versus cyclin D1 in control cultures. B, total RNA extracts from cells treated as in A were used for Northern blot analysis (top panels) and for reverse transcription-PCR for cyclin D1 (bottom panels), as described under “Experimental Procedures.” Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 28 S RNA were used as RNA loading controls. After withdrawal of Tet for the indicated times, asynchronously growing MCF7/BAD cells were kept in cysteine/methionine-free medium for 1 h and labeled with trans-[35S]Cys/Met (75 Ci/ml) for 2 h. Cyclin D1 immunoprecipitates were resolved on 12% SDS-PAGE. Band intensity is used as a measure of cyclin D1 de novo protein synthesis. Extracts from unlabeled cultures treated in parallel were used for Western blot in the bottom panel. Autoradiograms shown in this and other figures are representative of three separate experiments.

FIGURE 3. BAD RNAi regulates the cell cycle and cyclin D1 protein. A, asynchronously growing MCF7 cells were treated with BAD siRNA or control siRNA for 48 h. DNA synthesis was assayed by bromodeoxyuridine labeling for an additional 12 h as a measure of S phase fraction (n = 4 experiments, with 3 replicates in each experiment; ***, p < 0.001; bars, ± S.E.). Western blot analysis verified down-regulation of BAD in these cultures as in B. B, asynchronously growing MCF7 cells were treated with BAD siRNA or control siRNA for the indicated times, and protein expression was analyzed by Western blot. Same blots were stripped and probed for indicated molecules (note the relative decrease in actin at 48 and 72 h similar to that of BCLxL). C, densitometric analysis of cyclin D1 and BAD expression from Western blots of three independent experiments is shown in the graph. *, p < 0.05 for cyclin D1 (72 h) versus cyclin D1 (control). **, p < 0.01 for BAD (72 h) versus BAD (control). Bars, ± S.E.
data suggest that BAD may be found in complexes with DNA-bound AP1 proteins. Accordingly, when BAD expression is inhibited with siRNA, AP1-DNA interaction is increased above the basal level (Fig. 5B, compare lane 2 versus lane 4). This provides further evidence for interaction of endogenous BAD with AP1 complexes modulating AP1/DNA interactions, which may in turn manifest as decreased cyclin D1 expression and cell cycle blockade. In contrast to TRE regulation, neither BAD overexpression nor BAD RNAi had any effect on nuclear factor interactions with the NFκB-binding element (Fig. 5B, lanes 5–8), suggesting that interaction of BAD with AP1 proteins is specific.

Since c-Jun is one of the nuclear proteins that bind to the TRE and CRE elements and participates in AP1-dependent cyclin D1 transcription (27–29), we sought to determine whether BAD interacts with c-Jun. Endogenous BAD complexed with c-Jun in the nucleus but not in the cytoplasm of proliferating MCF7 cells (Fig. 5C, 1). Immunoprecipitation with increasing quantities of BAD antibody precipitated increasing quantities of c-Jun (Fig. 5C, 2, note that antibody concentrations in 1/H11003 BAD equal that in rabbit IgG). BCL2 did not complex with c-Jun under these same conditions, demonstrating the specificity of BAD and c-Jun interaction. As transcriptional functions of c-Jun are regulated by its phosphorylation status, we determined whether BAD/c-Jun interaction depends upon c-Jun phosphorylation by measuring active c-Jun (phosphorylated at Ser63 and Ser73 residues) in BAD immunoprecipitates. Only unphosphorylated c-Jun was detected in immunoprecipitates from nuclear extracts (Fig. 5D) for endogenous and exogenous BAD (FLAG-tagged). Importantly, BAD RNAi blocked demonstrable c-Jun binding by endogenous BAD (Fig. 5D), further confirming that endogenous BAD binds to c-Jun. Western blotting for c-Jun (inset) shows the efficient detection of phosphorylated c-Jun in these cells with the same anti-phospho-c-Jun antibody. A small quantity of BAD was detected in immunoprecipitates of c-Jun binding with endogenous BAD (Fig. 5D).
**Cell Cycle Functions of BAD**

**A**

| No NE | Con NE or BAD | BAD siRNA NE | BAD overexpressing |
|-------|---------------|--------------|--------------------|
| AP-1/TRE |                |              |                    |
| 1      | 2             | 3            | 4                  |
| Shift  | C-Jun         | BAD          | Tubulin            |
| S/Shift|                |              |                    |

**B**

| No NE | Con NE or BAD | BAD siRNA NE | BAD overexpressing |
|-------|---------------|--------------|--------------------|
| AP-1/TRE |                |              |                    |
| 1      | 2             | 3            | 4                  |
| Shift  | C-Jun         | BAD          | Tubulin            |
| S/Shift|                |              |                    |

**C**

Panel 1

| BAD IP | BAD | CYC | WCL |
|--------|-----|-----|-----|
| C-Jun  |     |     |     |

Panel 2

| IP with | 1X BAD IgG | 3X BAD IgG |
|---------|------------|------------|
| BAD     | 1         |            |

**D**

Panel 3

| IP of | BAD | phos c-Jun | C-Jun |
|-------|-----|------------|-------|
| 1     | 2   |            |       |

Panel 4

| IP of | BAD | C-Jun |
|-------|-----|-------|
| 1     | 2   |       |

**F**

Nuclear extracts were analyzed by Western blotting for BAD expression. Phos BAD S75 and Phos BAD S99 were used to detect phosphorylated BAD. E2F was used as a nuclear marker.

**G**

Endogenous BAD (25 kDa) was detected by Western blotting. Exogenous BAD, Phos BAD S75, and Phos BAD S99 were used for analysis.

**FIGURE 5.** BAD regulates AP1 protein interaction with their targets. A, for the EMSAs, the radiolabeled AP1/TRE sequence (TGACTCA) was incubated as denoted. Lane 2, control nuclear extract (NE); lane 3, BAD-depleted nuclear extract; lane 4, BCL2-depleted nuclear extract; lane 6, c-Jun-depleted nuclear extract; lane 7, unidentified (cold) nonspecific probe; lane 8, labeled (cold) specific probe. For the supershift analysis, control nuclear extract was incubated with c-Jun antibody (lane 8) or the BAD antibody (lane 9). Reactions were resolved by nondenaturing gel electrophoresis, and autoradiograms of the dried gels were obtained. Data shown are representative of three independent experiments. Band densities were measured using the ImageJ program, and densities obtained were compared with control nuclear extract (taken as 1.0): BAD-depleted, 0.78 (S.E. = 0.045, p = 0.01); c-Jun-depleted, 0.70 (S.E. = 0.032, p < 0.01); BCL2-depleted, 1.14 (S.E. = 0.22; not significant). p values were obtained as described under “Experimental Procedures.” In the right panel, a Western blot demonstrates the efficiency of depletion of the molecules from the nuclear extract. Tubulin is used as a protein loading control for the Western blot. B, MCF7 cell nuclear extracts of control (lanes 2 and 6), BAD overexpressing (lanes 3 and 7), and BAD siRNA-treated cells (lanes 4 and 8) were used in EMSA. Labeled AP1/TRE or labeled NF-kB binding element were used as probes. Reactions were subjected to electrophoresis and analyzed as in B. The right panel shows BAD expression levels after treatments. C, panel 1, BAD was immunoprecipitated (IP) from the cytosolic and nuclear fractions and from the whole cell lysate (WCL) of MCF7 cells. Immune complexes were resolved by SDS-PAGE. The presence of c-Jun was detected by Western blotting. A Western blot also verified the presence of E2F1 (a nuclear marker) in the nuclear and whole cell lysate. Panel 2, BAD, BCL2, and c-Jun were immunoprecipitated from 50 μg of MCF7 nuclear extracts. Rabbit immune serum was used as the control antibody. Anti-BAD IgG concentrations are denoted as 1X, 2X, and 3X, where X = 0.5 μg. All other antibodies used at 0.5 μg. Immune complexes were resolved by SDS-PAGE, and c-Jun was detected by Western blotting. Ten percent of nuclear extract used in immunoprecipitation was utilized as the input control. D, BAD or FLAG were immunoprecipitated from nuclear extracts of untransfected, wtBAD-transfected, or BAD siRNA-treated cells. Immune complexes were resolved by SDS-PAGE, and the presence of phosphorylated and total c-Jun was detected by Western blotting. A Western blot (inset) verifies the detection of phosphorylated c-Jun in these cells by the same antibody. E, BAD or c-Jun were immunoprecipitated from nuclear extracts, and the complexes were resolved by SDS-PAGE. The presence of BAD was detected by Western blotting. F, nuclear extracts, cytoplasmic extracts, and whole cell lysates (WCL) of MCF7 cell were analyzed by Western blot for total and phosphorylated BAD expression. G, nuclear extracts of MCF7 cells transfected with control vector or various BAD constructs (wtBAD, BAD S75A, BAD S99A, and dmBAD) were analyzed by Western blot to detect phosphorylated and total BAD. The white arrow points to the exogenous BAD, and the black arrow points to endogenous BAD. Tubulin levels are shown as protein loading control.
cycle progression in MCF7 cells (Fig. 6B); however, dmBAD did not exert this repression effect on cell cycle progression. Basal and E$_2$-induced increases in cyclin D1 expression were inhibited by BAD (Fig. 6C). De novo synthesis of cyclin D1 (Fig. 6D) and the basal and E$_2$-induced cyclin D1 promoter-luciferase activity (Fig. 6E) were also inhibited by BAD overexpression. Similar results were obtained in T47D breast cancer cells. Taken together, these data suggest that BAD blocks mitogenic effects of E$_2$ at least partly by cyclin D1 repression. The ChIP data in Fig. 4C stand in complete agreement with this view.

**DISCUSSION**

During previous studies, we found that the overexpression of BAD in the absence of an apoptotic stimuli did not cause apoptosis (10) but inhibited cell proliferation. In this study, we have determined the mechanism of this effect in detail using MCF7 cells, a well established cell culture model for hormone-responsive breast cancer. We have now discovered a novel role for BAD; this proapoptotic protein is able to inhibit cell cycle transit by enforcing a G$_1$-S block in MCF7 cells. This cell cycle-inhibitory role is independent of its proapoptotic role and is exerted at least partly through regulation of cyclin D1 synthesis. Cyclin D1 is a critical regulator of G$_1$ transit in breast cancer cells (11–13). The function of BAD as a cell cycle inhibitor does not require its dimerization domain but is dependent on phosphorylation at Ser$_{72}$ and Ser$_{99}$ residues and upon interaction with c-Jun.

In our experiments, we used three alternate approaches to demonstrate that BAD blocks MCF7 cell cycle in G$_1$: (a) transient transfection with a BAD-expressing vector, (b) stable overexpression of BAD; and (c) RNAi to inhibit expression of endogenous BAD. Stable overexpression of BAD inhibited cell growth but did not cause apoptosis under normal proliferative conditions. These cells accumulated in G$_1$ phase, and importantly, this arrest was reversible upon decrease in BAD expression. These observations confirm our previous work (10). Further, low levels of cyclin D1 mRNA and protein accompanied the decrease in the proliferative phase. Transient overexpression of BAD yielded similar results on cyclin D1 and cell cycle regulation. In agreement with the decrease in cyclin D1 expression, BAD overexpression drastically suppressed the de novo synthesis of cyclin D1 (Fig. 2). The repression of cyclin D1 was accompanied by decreased Cdk activities indicative of a G$_1$ block. Importantly, RNAi of endogenous BAD resulted in a higher proliferative fraction and increased cyclin D1, and the Cdk activities.
Our data also strongly suggest that BAD is able to decrease the activity of the cyclin D1 promoter in MCF7 and T47D breast cancer cells and that BAD exerts this effect most likely at the TRE and CRE element in the natural cyclin D1 promoter (Fig. 4). The ChIP assay demonstrates that E2 stimulation displaces endogenous BAD from the TRE and CRE motifs when it induces cyclin D1 expression. With the use of a minimal consensus TRE reporter construct we showed that BAD decreased activity at the AP1 binding element. Probably, BAD plays an indirect role in regulation of transcription at TRE/CRE elements in the cyclin D1 promoter. Our data show that c-Jun is likely to mediate this action, since BAD binds to nuclear c-Jun, and that this action is limited to unphosphorylated c-Jun. The basis of this observation is intriguing. It is possible that BAD sequesters c-Jun and prevents its activation by c-Jun N-terminal kinase or other mitogen-activated protein kinases. Such a mechanism may explain the ability of BAD to inhibit cyclin D1 induction, since the functions of AP1 proteins at the TRE and CRE in the cyclin D1 promoter will be blocked. Therefore, in analogy to a number of other nontranscriptional factors, BAD appears to modulate transcription by interacting with transcription factors.

Based on the data from the ChIP and EMSA assays, we propose that BAD interacts with c-Jun at the TRE/CRE motifs on the cyclin D1 promoter, forming a TRE/CRE c-Jun-BAD complex, which inhibits transactivation by c-Jun. Once E2 activates c-Jun N-terminal kinase and other mitogen-activated protein kinase pathways leading to c-Jun phosphorylation, BAD dissociates from the complex, and the TRE/CRE-phospho-c-Jun complex activates cyclin D1 transcription. Alternately, BAD may also bind to free c-Jun, and this dimer could bind to TRE/CRE motifs, inhibiting transcription. We should note that our data indicate both that BAD is present in complexes with c-Jun at the cyclin D1 promoter (CRE/TRE elements), and altering the levels of BAD expression through RNAi or enforced overexpression changes the evident TRE/CRE binding activity in nuclear extracts. This suggests alteration of (c-Jun-containing) complexes in such a way that DNA binding is diminished, although the exact nature of the respective (DNA) binding and nonbinding complexes is not known at this time. It is likely that there are equilibria between TRE/CRE-c-Jun-BAD, TRE/CRE-c-Jun, and BAD-c-Jun, and at any moment the relative proportion of these complexes is regulated by phosphorylation of BAD and c-Jun (excluding the complexities of c-Jun interactions with AP1 and CREB proteins). In effect, BAD may be acting to “titrate” c-Jun-dependent transcription at these elements.

In similarity to our studies on the subcellular distribution of phospho-BAD, other studies have failed to detect endogenous BID in the nuclear fraction, although this protein has a role in DNA damage response (1, 30). However, we clearly observed both BAD and phospho-BAD in ductal epithelium of the normal human breast (supplemental Fig. 3). This may suggest that phospho-BAD, but not BAD, somehow leaked out of the nuclear fraction of MCF7 cells during its preparation. To our knowledge, interaction of BCL2 family members with c-Jun has not been described before. The nearly simultaneous changes in cyclin D1 mRNA and protein following changes in BAD expression are consistent with changes in cyclin D1 previously described by us and others in MCF7 cells (11–13, 31–33).

A recent study showed that survival factors are less protective against insults when the Bad gene is knocked out in mice (34). Deletion of Bad led to development of malignancies of the hematopoietic system and shortened life span. Breast epithelial cells of these animals had lower rates of apoptosis but were refractory to growth factor-induced survival. Replacement of Bad with an unphosphorylatable form of Bad (where residues Ser75, Ser99, and Ser118 were mutated to alanine) increased the susceptibility to apoptosis in vitro and in vivo (35, 37). These studies confirmed the importance of BAD phosphorylation at these serine residues for its role in apoptosis. BAD-deficient mice displayed abnormal glucose metabolism resulting from the inability to form the enzyme (hexokinase II) complex. Transgenic mice expressing the unphosphorylatable Bad showed defective glucose metabolism, although the enzyme complex was assembled normally (36). This finding again suggests that phosphorylation and/or events secondary to phosphorylation are important for the “metabolic” functions of BAD.

Our results suggest that the Ser75 and Ser99 residues of BAD are important for its cell cycle effects, as clearly indicated by the inability of dmBAD to inhibit either cyclin D1 promoter activity or synthesis of cyclin D1 protein (Fig. 4A and supplemental Fig. 2C). In addition, we have observed that BAD/c-Jun interaction required Ser75 and Ser99 residues of BAD.4 Growth-promoting (38) and newly described survival (3) functions of BAD are dependent on Ser99 phosphorylation. Therefore, our data are consistent with prior findings that Ser75/Ser99 phosphorylation sites of BAD are required for its nonapoptotic functions. BH3 domain-only proapoptotic proteins, such as BID, are now known to have other types of cellular functions, such as a role in the DNA damage response (1, 30). Our data using BH3 deletion mutants suggest that dimerization with other BCL2 family members is not required for transcriptional functions of BAD. BAD, like other proapoptotic BCL2 family proteins is not simply a monofunctional protein (see Introduction). Indeed, BCL2 family proteins are multifunctional, and their splice variants, isoforms, and post-translational modifications determine the proapoptotic or prosurvival roles (see discussion in Ref. 3).

Our study presents detailed evidence for an exciting new role for BAD in breast cancer cell growth and proliferation. These data extend previous reports that proapoptotic proteins, such as BID (above) and BAX/BAK (39), have nonapoptotic functions. BAD appears to play a decisive role when breast cancer cells have to respond to mitogenic signals. Its negative influence on AP1-mediated cyclin D1 expression may play an important role in maintaining optimum level of cell cycle progression under normal proliferative conditions. The phosphorylated, inactivating residues of BAD are important in the cell cycle-regulatory role of BAD as positive regulatory elements in contrast to their negative regulatory role in apoptosis. The pathways, which phosphorylate these residues, are classically associated with growth/survival, and the ability of phospho-BAD to act as a cell cycle inhibitor may be viewed as a mechanism to restrict normal growth; perhaps this mechanism is subverted in tumor cells. In a larger context, our studies may also suggest that successful mitogenic
responses may require removal of negative regulatory activity exerted by BAD through mechanisms that dissociate BAD/c-Jun interactions.

REFERENCES

1. Zinkel, S. S., Hurvo, K. E., Ong, C., Abtahi, F. M., Gross, A., and Korsmeyer, S. J. (2005) Cell 122, 579–591
2. Dumay, A., Laulier, C., Bertrand, P., Saintigny, Y., Lebrun, F., Vayssiére, J. L., and Lopez, B. S. (2006) Oncogene 25, 3196–3205
3. Seo, S. Y., Chen, Y. B., Ivanovska, I., Ranger, A. M., Hong, S. J., Dawson, V. L., Korsmeyer, S. J., Bellsow, D. S., Fannjiang, Y., and Hardwick, J. M. (2004) J. Biol. Chem. 279, 42240–42249
4. Crescenzi, E., and Palumbo, G. (2001) Gynecol. Oncol. 81, 184–192
5. Deng, X., Gao, F., Flagg, T., and May, W. S., Jr. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 153–158
6. Chattopadhyay, A., Chiang, C. W., and Yang, E. (2001) Oncogene 20, 4507–4518
7. Letai, A., Baksa, M. K., Walensky, L. D., Soricelli, M. D., Weiler, S., and Korsmeyer, S. J. (2002) Cancer Cell 2, 183–192
8. Kelekar, A., Chang, B. S., Harlan, J. E., Fesik, S. W., and Thompson, C. B. (1997) Mol. Cell. Biol. 17, 7040–7046
9. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
10. Fernando, R. I., and Wimalasena, J. (2004) Mol. Cell. Biol. 15, 3266–3284
11. Altucci, L., Addeo, R., Cicatiello, L., Dauvois, S., Parker, M. G., Truss, M., Beato, M., Sica, V., Brescian, F., and Weiz, A. (1996) Oncogene 12, 2315–2324
12. Foster, J. S., and Wimalasena, J. (1996) Mol. Endocrinol. 10, 488–498
13. Pratt, O. W., Jr., Sarcevic, B., Musgrove, E. A., Watts, C. K. W., and Sutherland, R. L. (1997) J. Biol. Chem. 272, 10882–10894
14. Alle, K. M., Henshall, S. M., Field, A. S., and Sutherland, R. L. (1998) Clin. Cancer Res. 4, 847–854
15. Barnes, D. M., and Gillett, C. E. (1998) Breast Cancer Res. Treat. 52, 1–15
16. Lee, R. J., Albanese, C. F., Fu, M. F., D’Amico, M. F., Lin, B. F., Watanabe, G. F., Haines, G. K., III, Siegel, P. M., Hung, M.-C., Yarden, Y. F., Horowitz, J. M., Muller, W. J., and Pestell, R. G. (2000) Mol. Cell. Biol. 20, 672–683
17. Hirai, I., and Wang, H. G. (2001) Biochem. J. 359, 345–352
18. Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001) Nature 414, 313–317
19. Wadman, I. A., Osada, H., Gruitz, G. G., Agulnic, A. D., Westphal, H., Forster, A., and Rabbits, T. H. (1997) EMBO J. 16, 3145–3157
20. Foster, J. S., Henley, D. C., Bukovsky, A., Seth, P., and Wimalasena, J. (2001) Mol. Cell. Biol. 21, 794–810
21. Foster, J. S., Henley, D. C., Ahamed, S., and Wimalasena, J. (2001) Trends Endocrinol. Metab. 12, 320–327
22. Kitagawa, M., Higashi, H., Jung, H. K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., Kato, J., Segawa, K., Yoshida, E., Nishimura, S., and Taya, Y. (1996) EMBO J. 15, 7060–7069
23. Campo, P. A., Das, S., Hsiang, C. H., Bui, T., Samuel, C. E., and Straus, D. S. (2002) Cell Growth Differ. 13, 409–420
24. Watanabe, G., Howe, A., Lee, R. J., Albanese, C., Shu, I. W., Karnezis, A. N., Zon, L., Kyrkiakis, J., Rundell, K., and Pestell, R. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12861–12866
25. Liu, M. M., Albanese, C., Anderson, C. M., Hilty, K., Webb, P., Uht, R. M., Price, R. H., Jr., Pestell, R. G., and Kushner, P. J. (2002) J. Biol. Chem. 277, 24353–24360
26. Novick, N., Becker, E. B., Konishi, Y., and Bonni, A. (2002) J. Biol. Chem. 277, 40944–40949
27. Shaulian, E., and Karin, M. (2001) Oncogene 20, 2390–2400
28. Shaulian, E., and Karin, M. (2002) Nat. Cell Biol. 4, E131–E136
29. Wixson, R., Johnson, R. S., and Moore, C. (1999) EMBO J. 18, 188–197
30. Kamer, I., Sarig, R., Zaltsman, Y., Niv, H., Oberkowitz, G., Regev, L., Haimovich, G., Lenthal, Y., Marcellus, R. C., and Gross, A. (2005) Cell 122, 593–603
31. Lai, A., Sarcevic, B., Pratt, O. W., and Sutherland, R. L. (2001) J. Biol. Chem. 276, 25823–25833
32. Dees, C., Askari, M., Foster, J. S., Ahamed, S., and Wimalasena, J. (1997) Mol. Carcinog. 18, 107–114
33. Ahamed, S., Foster, J. S., Bukovsky, A., and Wimalasena, J. (2001) Mol. Carcinog. 30, 88–98
34. Ranger, A. M., Zha, J., Harada, H., Datta, S. R., Danial, N. N., Gilmore, A. P., Kurtok, J. L., Le Beau, M. M., Greenberg, M. E., and Korsmeyer, S. J. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9324–9329
35. Datta, S. R., Ranger, A. M., Lin, M. Z., Sturgill, J. F., Ma, Y. C., Cowan, C. W., Dikkes, P., Korsmeyer, S. J., and Greenberg, M. E. (2002) Dev. Cell 3, 631–643
36. Danial, N. N., Gramm, C. F., Scorrano, L., Zhang, C. S., Krauss, S., Ranger, A. M., Datta, S. R., Greenberg, M. E., Licklider, L. J., Lowell, B. B., Gygi, S. P., and Korsmeyer, S. J. (2003) Nature 424, 952–956
37. Bonni, A., Brunet, A., West, A. E., Datta, S. R., Takasu, M. A., and Greenberg, M. E. (1999) Science 286, 1358–1362
38. Maslyar, D. J., Aoki, M., and Vogt, P. K. (2001) Oncogene 20, 5087–5092
39. Hetz, C., Bernasconi, P., Fisher, J., Lee, A. H., Bassik, M. C., Antonsson, B., Brandt, G. S., Ikwoshi, N. N., Schinzler, A., Glimcher, L. H., and Korsmeyer, S. J. (2006) Science 312, 572–576
40. Musgrove, E. A., Swarbrick, A., Lee, C. S., Cornish, A. L., and Sutherland, R. L. (1998) Mol. Cell. Biol. 18, 1812–1825
41. Pratt, O. W., Rogan, E. M., Musgrove, E. A., Watts, C. K., and Sutherland, R. L. (1998) Mol. Cell. Biol. 18, 4499–4508