Epidermal Growth Factor Protects Prostate Cancer Cells from Apoptosis by Inducing BAD Phosphorylation via Redundant Signaling Pathways

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Protection from apoptosis by receptor tyrosine kinases, resistant to the inhibition of phosphatidylinositol 3′-kinase/Akt and Ras/MEK pathways, has been reported in several cell types, including fibroblasts and epithelial prostate cancer cells; however, mechanisms of this effect were not clear. Here we report that in prostate cancer cells, epidermal growth factor activates two antiapoptotic signaling pathways that impinge on the proapoptotic protein BAD. One signaling cascade operates via the Ras/MEK module and induces BAD phosphorylation on Ser112. Another pathway predominantly relies on Rac/PAK1 signaling that leads to BAD phosphorylation on Ser136. Each of these two pathways is sufficient to protect cells from apoptosis, and therefore both have to be inhibited simultaneously to block epidermal growth factor-dependent survival. Redundancy of antiapoptotic signaling pathways should be considered when therapies targeting antiapoptotic mechanisms are designed.

Since apoptosis was identified as one of the mechanisms of cancer cell elimination (1, 2), substantial efforts were spent to understand regulation of this process at the molecular level. These efforts revealed that the decision to undergo apoptosis is determined by external or intrinsic pro- and antiapoptotic stimuli (3). It became evident that antiapoptotic mechanisms not only help resisting anti-cancer therapies but are also a prerequisite for oncogenic transformation itself. Thus, the ability to avoid apoptosis was recognized as an indispensable component of cancer cells (4, 5).

Prostate cancer provides an excellent illustration of the significance of antiapoptotic signaling for tumorigenesis. Prostate epithelial cells depend on androgen for their growth and survival and undergo apoptosis when androgen levels are decreased (6). Androgen dependence of prostate cells forms the basis for the treatment of prostate cancer. Unfortunately, in many instances, androgen ablation therapy fails because prostate cancer cells engage antiapoptotic mechanisms that allow them to survive without androgen (7–9).

One of the first discovered and most extensively characterized anti-apoptotic pathways is the PI3K/Akt module (10). The constitutive activation of the PI3K signaling pathway has been found in a variety of cancers, including advanced prostate cancer. This activation often occurs due to a loss of expression of the tumor suppressor gene PTEN, which encodes a lipid phosphatase that negatively regulates signaling pathways downstream of PI3K (11, 12). Knock-out of the PTEN gene in the mouse prostate is sufficient to trigger development of metastatic prostate cancer (13). These observations established the importance of PI3K signaling in the pathogenesis of prostate cancer. In addition to PI3K, other antiapoptotic signaling pathways may become activated in prostate cancer cells (7).

The increased expression of EGFR and its ligands has been observed in prostate cancer samples for some time (14, 15), but the role of the EGFR axis in apoptosis regulation in prostate cells only recently became appreciated. Thus, several investigators have reported that EGF can protect prostate cancer LNCaP cells from apoptosis induced by PI3K inhibitors; however, signal transduction pathways as well as targets of EGF-activated survival remained arcane (16, 17).

Previously EGF-induced suppression of apoptosis has been observed in several tissue culture cell lines, as well as in model organisms (18–21). In each case, inhibition of individual survival signaling pathways downstream of EGFR would diminish the antiapoptotic effects of EGF (22–25).

Here we demonstrate that EGF activates two parallel antiapoptotic signaling pathways that impinge on BAD in androgen-dependent LNCaP and androgen-independent C4-2 prostate cancer cells. One signaling cascade operates via the Raf/MEK module and induces phosphorylation of Ser112. Another pathway predominantly relies on Rac/PAK1 pathway and leads to phosphorylation of Ser136. Each of these two pathways is sufficient to protect from apoptosis, and therefore both signaling pathways must be inhibited to sensitize prostate cancer cells to proapoptotic treatments. Thus, redundancy of antiapoptotic signaling pathways activated by single survival agonist should

2 The abbreviations used are: PI3K, phosphatidylinositol 3′-kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MEK, mitogen activated protein kinase kinase; MSK1, mitogen- and stress-activated protein kinase 1; CREB, cAMP-responsive element binding protein; PAK, p21-activated kinase; PTEN, phosphatase and tensin homolog deleted on chromosome ten; 4HT, 4-OH tamoxifen; HA, hemagglutinin; GFP, green fluorescent protein.
**EGF Induces BAD Phosphorylation via Raf and Rac**

be considered when therapies targeting antiapoptotic mechanisms are designed.

**EXPERIMENTAL PROCEDURES**

**Cell Line**—The prostate cancer LNCaP and C4-2 cells were a gift from Leland Chung. C4-2LucBAD cells were obtained by transfecting C4-2 cells by pGL4 mixed with Tet_on and pTRE2hygBAD cDNA constructs. Cells were selected in G418 and hygromycin antibiotics. 60 clones were isolated and further transfecting C4–2 cells by pGL4 mixed with Tet_on and gift from Leland Chung. C4-2LucBAD cells were obtained by pTRE2hygBAD cDNA constructs. Cells were selected in G418 and hygromycin antibiotics. 60 clones were isolated and further

**Antibodies and Other Reagents**—Antibodies were from the following sources: BAD, phospho-specific BAD (Ser112, Ser136, and Ser157), phospho-ERK (Thr202/Tyr204), phospho-Akt (Ser473), p90rsk (Thr360/Ser364), PTEN, and phospho-CREB (Ser133) were from Cell Signaling Technology. Antibodies were from Sigma; secondary horseradish peroxidase-conjugated antibodies used for Western blots were from Amersham Biosciences. Protein G-agarose beads, PD98059, DEVD-AMC, and rapamycin were from Calbiochem. Recombinant EGF was from Upstate Biotechnology, Inc. (Lake Placid, NY). All other chemicals and reagents (unless specified) were purchased from Sigma. Tissue culture reagents were purchased from Invitrogen.

**Plasmid and DNA Constructs**—pcDNA3-3HA-BAD and pcdNA3-HA-BADs112/136A constructs were described by Robert Datta and Michael Greenberg (Harvard Medical School, Boston, MA); pEF-Myc-N17Rac2 and pEF-Myc-N17Cdc42 constructs were from Andrew Thorburn (University of Colorado, Denver, CO); pkH3-HA-Rsk1(K112R/K464R) was from John Blenis (Harvard Medical School, Boston, MA); pCMV14-FLAG-CREB was from Gary Kammer (Wake Forest University School of Medicine); pRK5-Myc-PAC1(K299R), pCMV-HA-MEK1, and pCMV-HA-MEK1(S218/222A) were described by Andrew Catling (Louisiana State University, New Orleans, LA). The pBabeeno3ΔRaf1-ER[DD] (ΔRaf1-1:ER) and pLNC-Raf324-FLAG (C4BRaf) were from Martin McMahon (University of California, San Francisco, CA). PTEN was from Liwu Li (Virginia Tech, Blacksburg, VA). GFP-DEVDP-BFP (pGdB) was from Rigel Inc. (Sunnyvale, CA). EGFP was from Clontech (Mountain View, CA).

**Cell Culture and Transfection**—LNCaP and C4-2 cells were plated in 10-cm plates and maintained in T-medium supplemented with 5% fetal bovine serum and RPMI 1640 with 10% fetal bovine serum, respectively. All cells were kept in 5% CO2 at 37 °C. Transient transfection was performed by using Lipoctene (Invitrogen) according to the manufacturer’s recommendations. The total amount of transfected DNA was maintained at 0.6 μg with an empty vector in the 10-cm plate. In most experiments, the following amounts of DNA were used per one 10-cm plate: 0.6 μg of EGFP, 0.5 μg of BAD, 0.5 μg of BclXL, and 4 μg of C4BRaf, RafER, DN-MEK1, DN-p90rsk, DN-PACK1, DN-Rac, or DN-Cdc42 expression constructs. In co-transfection experiments where the combined effects of C4BRaf and DN-PACK1 on BAD phosphorylation were studied, the cells were transfected with 0.6 μg of BAD, 0.6 μg of BclXL, and 2.4 μg each of C4BRaf and/or DN-PACK1. In experiments where apoptosis was measured, the cells were transfected as described above, but BclXL was replaced with 0.3 μg of EGFPP.

**Immunoprecipitation**—20 h after transfection, cells were deprived of serum for 3 h, and different treatments were given at this point. Cells were harvested in a cell lysis buffer (20 mM Tris, pH 7.4, 40 mM NaF, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 μg each of leupeptin, pepstatin, and aprotonin, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO4, 50 mM β-glycerophosphate, 40 mM p-nitrophenyl phosphate, and 1 mM dithiothreitol). The lysates were cleared of insoluble material by centrifugation at 14,000 × g for 10 min at 4 °C. Cell extracts were incubated with 6–8 μg of anti-HA antibodies (12CA5) overnight at 4 °C and protein A-conjugated beads for another 3 h. Beads were washed three times with cell lysis buffer, and proteins were eluted with an SDS sample buffer for Western blotting analysis.

**Apoptosis Assays**—Apoposis in a population of transiently transfected GFP-positive cells was measured by time lapse video recording followed by counting the percentage of cells with apoptotic morphology. For these experiments, cells were plated into 6-well plates and transfected by Lipofectamine with a mixture of cDNA that included GFP in a 1:10 ratio. 20 h after transfection, cells were placed in serum-free medium for another 20 h and treated with 50 μM LY294002 and 1 μM thapsigargin to induce apoptosis. 15 min later, cells were treated with EGF or 4-OH tamoxifen (4HT). Video recording was performed on an Axiovert100 microscope (Carl Zeiss) equipped with a moving stage and climate control chamber (37 °C, 5% CO2 and controlled by Openlab software (Improvision Inc., Lexington, MA). At least four randomly chosen fields containing 250–350 cells for each treatment were recorded.

Apoptosis in fragmented cells was confirmed by immunofluorescent detection of active caspase 3 and nuclear fragmentation. For this purpose, cells were fixed with 4% paraformaldehyde for 20 min and stained with antibodies to active caspase 3 (Idun Pharmaceuticals, La Jolla, CA), followed by goat anti-rabbit antibodies conjugated with CY5 (Jackson Laboratories, West Grove, PA) and 0.2 μg/ml 4′,6-diamidino-2-phenylinole. Then cells were rinsed in distilled water and mounted using Vectashield (Vector Laboratories, Burlingame, CA). Fluorescent and phase-contrast images were taken on an Olympus microscope equipped with digital camera and software from Media Cybernetics Inc., Silver Spring, MD).

Apoptosis in whole cell populations was quantitated by measuring caspase 3 activity with the fluorogenic substrate Ac-DEVDP-7-amido-4-trifluoromethylcoumarin (Bachem) as specified by the manufacturer. For these experiments, attached and floating cells were collected and lysed in caspase lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, and 5 μg/ml aprotonin, leupeptin, and pepstatin). Fluorescence was recorded each 15 min for 1 h, and caspase activity was expressed in arbitrary units. Presented results were confirmed by at least two independent experiments.
RESULTS

Tyrosine Kinase Activity of EGFR Is Required for EGF-induced BAD Phosphorylation and Cell Survival—Previously, we reported that EGF delays apoptosis in LNCaP cells in the presence of PI3K inhibitors and cycloheximide (16). Recently, we have demonstrated that BAD phosphorylation was necessary for antiapoptotic effects of EGF. BAD2SA where Ser112 and Ser136 were replaced with alanines behaved as a dominant negative mutant that inhibited the antiapoptotic effect of EGF (26). However, the signaling mechanism of EGF-induced BAD phosphorylation was not clear.

The proapoptotic function of BAD is inhibited by phosphorylation at Ser112, Ser136, and Ser155 (based on the mouse sequence), whereas dephosphorylation of BAD promotes apoptosis (27). Phosphorylation of endogenous BAD in LNCaP cells can be detected with phospho-specific antibodies raised against Ser112; however, phospho-specific antibodies to Ser136 and Ser155 are not sensitive enough to detect phosphorylation of endogenous BAD in whole cell lysates. Therefore, analysis of BAD phosphorylation was conducted in cells that ectopically express HA-BAD. Time course analysis of BAD phosphorylation demonstrated that endogenous and ectopically expressed proteins underwent similar changes in cells treated with combinations of LY294002 and EGF; the addition of LY294002 resulted in continuous dephosphorylation of BAD, whereas EGF restored BAD phosphorylation (Fig. 1, A and B). In these experiments, HA-BAD was expressed at a 1:4 ratio to endogenous BAD in order to avoid overexpression-dependent artifacts (Fig. S1). Analysis of phosphorylation of immunoprecipitated HA-BAD showed that after treatment with LY294002, BAD became dephosphorylated at Ser112 and Ser136, whereas treatment with EGF restored phosphorylation at both sites (Fig. 1B and Fig. S2). Treatment of LNCaP cells with EGF induced activation of ERK1/2 but did not restore Akt phosphorylation in the presence of LY294002. Comparable effects of EGF on BAD and ERK phosphorylation were observed in cells maintained in the presence of serum (Fig. S3).

Parallel with BAD phosphorylation, we analyzed apoptosis in cells with endogenous BAD levels as well as in cells that ectopically express HA-BAD. In agreement with our previous results, EGF decreased apoptosis in LNCaP cells with endogenous BAD levels (Fig. 1C). Cells that ectopically expressed HA-BAD underwent apoptosis faster, but the antiapoptotic effect of EGF was still evident in these cells (Fig. 1C). Apoptosis in these experiments was measured by time lapse video recording that allows us to follow apoptosis in individual cells. Cells with apoptotic morphology were also recognized with antibodies to active caspase 3 (Fig. 1D). Time lapse results were further confirmed by caspase assays that allow quantification of apoptosis in a whole cell population. Caspase assays showed that EGF decreased apoptosis in LNCaP as well as in C4-2 cells characterized by higher tumorigenic potential. Consistent with the results of time lapse video recording in LNCaP cells that transiently express HA-BAD, increased apoptosis was observed in C4–2LucBAD cells that stably express HA-BAD (Fig. 1E).

To characterize the mechanism utilized by EGF to induce BAD phosphorylation and to protect LNCaP cells from apoptosis, we began by testing whether tyrosine kinase activity of the EGF receptor is necessary for BAD phosphorylation and survival.

LNCaP cells were treated with 10 μM AG1478, a selective inhibitor of EGFR tyrosine kinase, and BAD phosphorylation was followed by Western blotting. AG1478 blocked the ability of EGF to activate EGFR (Fig. S4A) and inhibited EGF-induced phosphorylation of BAD at both Ser112 and Ser136 in cells treated with PI3K inhibitor (Fig. S4B). Similarly, the antiapoptotic effect of EGF was completely blocked in the presence of AG1478 (Fig. S4C). These results indicate that EGFR tyrosine kinase activity is necessary for protection from apoptosis and BAD phosphorylation in prostate cancer cells.

Ras/Raf Signaling Induces Phosphorylation of Ser112 BAD—Ras activation plays an important role in proliferative and survival signaling downstream from receptor tyrosine kinases (28). Raf kinases are identified as immediate downstream effectors of Ras transmitting signal to ERK1/2 kinases. Because there was a strong correlation between activation of ERK1/2 and BAD phosphorylation induced by EGF (Fig. 1A), we began from analysis of this pathway. To address the role of the Ras/Raf signaling module in BAD phosphorylation, we took advantage of inhibitory and conditionally activated mutants of C-Raf. The C4B-Raf protein contains only the N-terminal regulatory domain of the C-Raf protein that interacts with the Ras, displaces endogenous Ras effectors, and therefore blocks Ras function (29).

First the effects of dominant negative C4B-Raf on BAD phosphorylation stimulated by EGF were examined. LNCaP cells were co-transfected with C4B-Raf and HA-BAD, and BAD phosphorylation was analyzed after treatments with LY294002 and EGF. The C4B-Raf significantly inhibited EGF-induced Ser112 phosphorylation (Fig. 2A, compare lanes 3 and 6); however, Ser136 phosphorylation was only slightly diminished. Constitutive Akt phosphorylation was unaffected by C4B-Raf, suggesting the absence of the cross-talk between Raf and PI3K/Akt signaling pathways in LNCaP cells (Fig. S5).

In order to test if the activation of Raf kinase is sufficient to induce BAD phosphorylation, we used a ΔRaf-1:ER construct (30). Deletion of the regulatory domain of Raf-1 generates a constitutively active kinase. Fusion of this deletion mutant to the hormone-binding domain of the human estrogen receptor creates a chimeric kinase (ΔRaf-1:ER) that is kept in the inactive state bound to heat shock proteins and can be rapidly activated by the estradiol analog 4HT. LNCaP cells were co-transfected with ΔRaf-1:ER and HA-BAD, and BAD phosphorylation was examined. The addition of 1 μM 4HT to the cells expressing ΔRaf-1:ER induced robust phosphorylation of BAD at Ser112 (Fig. 2A, lane 9). In sharp contrast, the Ser136 BAD phosphorylation was not induced by activated Raf-1 kinase.

Since Raf-1 was previously implicated in survival signaling (31, 32), we sought to determine whether Raf-1 activation alone is sufficient to protect LNCaP cells from LY294002-induced apoptosis. Cells were transfected with a mixture of cDNA encoding ΔRaf-1:ER and GFP, and the apoptotic cell death of GFP-positive cells was followed by time-lapse video recording. As shown in Fig. 2B, both EGF and 4HT inhibited apoptosis with similar efficacies.
**FIGURE 1. BAD phosphorylation correlates with apoptosis in prostate cancer cells.**

A, time course of endogenous BAD phosphorylation in LNCaP cells. Cells were serum-starved and treated with LY294002 (LY) followed with EGF 2 h later. Incubation was continued for the indicated time, and then cells were lysed and immunoblotted for phospho-Ser473 Akt, phospho-Thr308 Akt, total Akt, phospho-ERK1/2, total ERK1/2, phospho-Ser112 BAD, and total BAD. Inhibition of Akt activity in cells treated with LY294002 was confirmed by measuring phosphorylation of GSK3 peptide added to Akt immunoprecipitates (not shown).

B, LY294002 induces dephosphorylation of HA-BAD at Ser112 and Ser136. LNCaP cells were transfected with HA-BAD and FLAG-BclXL. 20 h after transfection, cells were serum-starved for 3 h and treated with LY294002 for indicated time. Cells were lysed, and HA-BAD was immunoprecipitated (IP) using anti-HA antibodies. Phosphorylation of BAD was followed by Western blot analysis with antibodies specific for Ser112, Ser136, and Ser155. Anti-BAD antibodies were used to confirm equal loading. Dephosphorylation of BAD was also observed in LNCaP cells, where HA-BAD was co-transfected with PTEN (Fig. S2).

C, time lapse analysis of apoptosis in LNCaP cells transiently transfected with HA-BAD. LNCaP cells were serum-starved and treated with 50 μM LY294002 and 1 μM thapsigargin (cells transfected with empty pcDNA3 vector and GFP) or LY294002 alone (cells transfected with HA-BAD and GFP), followed by 5 nM EGF. The percentage of apoptosis was determined by counting at least 350 cells in several randomly chosen fields for every treatment. D, GFP-positive (green) LNCaP cells with apoptotic morphology (cell fragmentation) show nuclear fragmentation and activation of caspase 3. 6 h after the induction of apoptosis, cells were fixed and analyzed for active caspase 3 by indirect immunofluorescence (red). Nuclear morphology was visualized by staining with 4',6-diamidino-2-phenylindole (DAPI) (blue). Microphotographs of the same field show phase-contrast and fluorescent images. The arrows point at GFP-positive cells. E, LNCaP, C4-2L, and C4-2LucBAD cells were treated as in C and collected by combining adherent and floating cells. Caspase activity in cell lysates was measured with the fluorescent substrate Ac-DEVD-7-amido-4-trifluoromethyloxycoumarin.
Results of apoptosis analysis by time lapse video recording were further confirmed by cleavage of caspase 3 substrate GFP-DEVD-BFP (pGDB). Treatment of LNCaP cells transfected with empty vector with EGF or cells transfected with ΔRaf-1:ER construct with 4HT reduced the appearance of cleaved product in cells incubated with LY294002 (Fig. 2C). Similar data were obtained in C4-2 cells.

In summary, EGF-induced phosphorylation of BAD at Ser\(^{112}\) is mediated via the Ras/Raf module. Activation of this signaling pathway alone is sufficient to protect against LY294002-induced cell death.

**MEK1 Inhibition Abrogates Ser\(^{112}\) Phosphorylation Induced by EGF**—MEK1 is a dual specificity kinase, which is activated by kinases of the Raf family and in turn activates ERKs (33). We next examined the role of MEK1 in the EGF-induced survival and phosphorylation of BAD. Treatment of LNCaP cells with a pharmacological inhibitor of MEK1 activation PD98059 prevented EGF-induced phosphorylation of Ser\(^{112}\) but did not affect Ser\(^{136}\) phosphorylation (Fig. 3A).

Analysis of apoptosis in cells treated with PD98059 shows a modest effect on EGF-stimulated survival, suggesting that BAD phosphorylation at Ser\(^{136}\) alone is sufficient to mediate the cytoprotective effect of EGF (Figs. 3B and S6).

Since PD98059 can inhibit MEK1, MEK2, and MEK5, we sought to determine which kinase is regulating Ser\(^{112}\) phosphorylation. For this purpose, we used a dominant negative MEK1(S218A/S222A) that specifically inhibits activation of ERK1/2 but not ERK5/big MAP kinase 1 (34). Cells were co-transfected with DN-MEK1 and HA-BAD, and BAD phosphorylation was examined. Ser\(^{112}\) phosphorylation induced by EGF was significantly reduced in cells expressing DN-MEK1, confirming that BAD phosphorylation at Ser\(^{112}\) is MEK1-dependent (Fig. 3C).

**BAD Phosphorylation at Ser\(^{112}\) Is Independent from p90\(^{rsk}\) and MSK1**—The kinases of the p90\(^{rsk}\) family have been reported to mediate BAD phosphorylation induced by antiapoptotic agonists downstream of MEK1 (35–37). We therefore speculated that p90\(^{rsk}\) might be the candidate kinase for Ser\(^{112}\) BAD phosphorylation induced by EGF. To test this hypothesis, we examined p90\(^{rsk}\) activation in LNCaP cells.
p90rsk possesses two kinase domains. When activities of these kinase domains are disabled by mutations in ATP binding loops, mutant p90rsk (K112R/K464R) acquires dominant negative properties. Indeed, a dominant negative effect of p90rsk (K112R/K464R) in LNCaP cells was clearly evident in the experiment that examined CREB phosphorylation induced by EGF (Fig. 4B). Next, we tested whether inhibiting p90rsk prevents BAD phosphorylation at Ser112. However, to our surprise, co-expression of p90rsk (K112R/K464R) with HA-BAD failed to inhibit EGF-induced phosphorylation at Ser112 (Fig. 4D). Taken together, these data indicate that EGF-induced phosphorylation of BAD at Ser112 is independent from p90rsk.

Another kinase downstream from ERK1/2 that potentially can phosphorylate BAD is mitogen- and stress-activated protein kinase-1 (MSK1) (38). Growth factors and stress signaling via ERK1/2 or p38 are known to activate MSK1, which in turn phosphorylates CREB and other substrates. Indeed, UV and EGF induced CREB phosphorylation in cells transfected with empty vector but not in cells where kinase-deficient MSK1(D195A) was co-transfected with CREB (Fig. 4C). To address the possible role of this kinase, we examined effects of MSK1(D195A) on BAD phosphorylation. In contrast to CREB phosphorylation, BAD phosphorylation induced by EGF was not inhibited by MSK1(D195A) (Fig. 4D). Thus, neither p90rsk nor MSK1 phosphorylates BAD at Ser112 downstream of the Ras/Raf/MEK/ERK cascade.

Expression of PAK1(K299R) Inhibits EGF-induced BAD Phosphorylation at Ser136—We then turned our attention to the signaling pathway that leads to phosphorylation of BAD at Ser136. So far, Akt, PAK1, and p70S6 kinases have been implicated in BAD phosphorylation at this site (39–42). EGF induces BAD phosphorylation in the presence of LY294002, which inhibits PI3K and Akt activity, suggesting that Akt is not responsible for this effect (see Fig. 1A). Likewise, EGF-induced phosphorylation of BAD remained intact regardless of the inhibition of p70S6 kinase phosphorylation by rapamycin (Fig. S7).

Next, we examined the role of PAK1 in BAD phosphorylation at Ser136. When activated, PAK1 phosphorylates MEK1 at Thr359/Ser363 (required for kinase activation) has shown that EGF activates p90rsk in an MEK-dependent fashion (Fig. 4A).

Analysis of p90rsk phosphorylation at Thr359/Ser363 (required for kinase activation) has shown that EGF activates p90rsk in an MEK-dependent fashion (Fig. 4A).
EGF Induces BAD Phosphorylation via Raf and Rac

Results of time lapse video recording were further supported by analysis of cleavage of caspase 3 substrate pGDB. Treatment with EGF inhibited cleavage of pGDB in cells transfected with either C4B-Raf or PAK1(K299R); however, it failed to do so in cells where both C4B-Raf and PAK1(K299R) were expressed simultaneously.

In summary, EGF protects prostate cancer cells by activating two signaling pathways that lead to BAD phosphorylation. One pathway is engaged through the classical Ras/Raf/MEK1 cascade and an unknown protein kinase that phosphorylates BAD at Ser<sup>112</sup>. Another signaling pathway that leads to Ser<sup>136</sup> is mediated by Rac/PAK signaling.

DISCUSSION

Ras/mitogen-activated protein kinase and PI3K/Akt signaling pathways have been shown to mediate transcriptionally independent anti-apoptotic effects of activated receptor tyrosine kinases (23, 47–49). Furthermore, several groups have reported that activation of receptor tyrosine kinases protects cells from apoptosis even when both PI3K and MEK signaling pathways are inhibited (36, 50, 51). These reports are consistent with our earlier findings in LNCaP cells. Thus, an antiapoptotic effect of EGF was evident in cells simultaneously treated with LY294002 and PD98059 (70) (Fig. 4B).

BAD phosphorylation was originally identified as a convergence point between PI3K/Akt and MEK/ERK/p90<sup>rsk</sup> protein kinase cascades activated by receptor tyrosine kinase and apoptosis machinery (35, 39, 52). Subsequently, several other protein kinases, including protein kinase A, p70S6, and PAK, were shown to exert their antiapoptotic function by phosphorylating BAD (40, 41, 53). All of these kinases were identified as distal components of linear survival pathways connecting receptor tyrosine kinase and apoptosis regulatory proteins. Inhibition of these kinases led to cessation of survival effects.

Analysis of EGF-induced protection from apoptosis of LNCaP cells has revealed an unusual organization of signaling pathways leading to BAD phosphorylation. EGF activates two signaling pathways downstream from EGFR; one pathway proceeds via the Ras/Raf/MEK cascade and results in the phosphorylation of BAD at Ser<sup>112</sup>; another pathway utilizes the Rac/PAK signaling module that leads to phosphorylation of BAD at Ser<sup>136</sup> (Fig. 8).

Inhibition of any single pathway connecting EGFR with BAD has only a small effect on apoptosis. Therefore, both pathways should be inhibited to ablate the antiapoptotic effect of EGF. Indeed, phosphorylation at either site is reportedly sufficient...
for 14-3-3 binding and, thus, inhibits proapoptotic function of BAD (54).

In this paper, a combination of the PI3K inhibitor LY294002 and thapsigargin (an inhibitor of the CERCA calcium pump that has recently emerged as a promising therapeutic agent for prostate cancer (55)) was used to trigger rapid apoptosis in LNCaP cells. Because we focused on antiapoptotic effects of BAD phosphorylation, a relatively swift post-translational response, we reasoned that a protocol that induced rapid apoptosis was most appropriate to demonstrate a connection between BAD phosphorylation status and apoptosis. Numerous downstream events triggered by EGF besides BAD phosphorylation at Ser112 and Ser136 may influence long term cell survival.

Recently, She et al. (21) demonstrated that in breast cancer cells EGF induces BAD phosphorylation at Ser\textsuperscript{112}, and PI3K signaling regulates Ser\textsuperscript{136} phosphorylation. Similar to our results, inhibition of both pathways was required for maximal apoptosis. Inhibition of any single pathway had a partial effect on apoptosis, although in breast cancer cells, the proapoptotic effect was stronger than we observed in prostate cells. What are the possible reasons for these discrepancies? In our paper, apoptosis assays were conducted 4 h after BAD dephosphorylation, whereas in the paper of She et al., apoptosis or survival was measured in 24–72 h (21). During this extended time, events other than BAD phosphorylation may contribute to apoptosis inhibition. Also, in breast cancer cells, other proteins may compete with BAD for 14-3-3 binding; therefore, only when both Ser\textsuperscript{112} and Ser\textsuperscript{136} are phosphorylated is BAD neutralized by 14-3-3.

It was suggested that Raf kinases can directly phosphorylate BAD and Bcl2 (32). In order to determine whether EGF-induced BAD phosphorylation is mediated by Raf itself or its downstream effectors, we performed experiments with the MEK inhibitor PD98059. These experiments show inhibition of EGF-induced BAD phosphorylation at Ser\textsuperscript{112} and thus exclude the possibility that Raf kinases phosphorylate BAD directly.

Since PD98059 can inhibit MEK1 and MEK5, it is possible that effects of EGF on BAD phosphorylation are mediated by ERK5/big MAP kinase, a downstream effector of MEK5 that was previously shown to phosphorylate BAD at Ser\textsuperscript{112} (56). Indeed, we observed activation of big MAP kinase in LNCaP cells treated by EGF (data not shown). However, experiments with dominant negative MEK1(S118A/S222A) that specifically inhibited ERK2 but not ERK5 (34) established the predominant role of signaling via MEK1 as an upstream kinase on a pathway leading to BAD phosphorylation at Ser\textsuperscript{112} (Fig. 3). Both Ser\textsuperscript{112} and Ser\textsuperscript{136} lie within the RXRXXpS motif (where pS represents

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phosphoserine), which can be recognized by ribosomal S6 and MSK kinases but not by MEK or ERK kinases. Therefore, in a subsequent set of experiments, we focused on the analysis of p90rsk and MSK1 as potential kinases responsible for EGF-induced phosphorylation of BAD.

p90rsk has been identified as the distal kinase of Ras-mitogen-activated protein kinase pathway responsible for Ser112 phosphorylation induced by brain-derived neurotrophic factor, 12-O-tetradecanoylphorbol-13-acetate, and interleukin-3 (35–37). MSK1 is known to phosphorylate BAD in UV-treated cells, but it can also be activated by EGF and potentially can mediate EGF-induced BAD phosphorylation as well. However, our experiments have shown that EGF-induced BAD phosphorylation at Ser112 is insensitive to the overexpression of a dominant negative mutant of either p90rsk1 or MSK1. Further experiments are needed to determine whether EGF-induced phosphorylation at Ser112 is mediated by a known or unknown kinase downstream of MEK1.

The second signaling pathway that connects EGFR and BAD operates independently from the Ras/MEK module. Previously, Akt, PAK1, and p70S6 kinases were shown to phosphorylate BAD at Ser136. Experiments with LY294002 and rapamycin (pharmacological inhibitors of PI3K and Raptor/mTOR) dismissed the Akt and p70S6, whereas dominant negative mutants of PAK1 and Rac inhibited EGF-induced phosphorylation of Ser136. The simplest explanation for these results is that EGF-induced phosphorylation at Ser136 is mediated by the Rac/PAK signaling pathway.

FIGURE 7. EGF-induced BAD phosphorylation and survival effect are inhibited by C4B-Raf and PAK1(K229R). A, BAD phosphorylation at both Ser112 and Ser136 is inhibited by C4B-Raf and DN-PAK1. Cells were transfected with HA-BAD, FLAG-BclXL, and either empty vector, C4B-Raf, PAK1(K229R), or both (see “Experimental Procedures” for details). 20 h after transfection, cells were serum-starved and treated with 50 μM LY294002 (LY) and 30 ng/ml EGF. Phosphorylation of immunoprecipitated HA-BAD was analyzed using phosphospecific antibodies as in Fig. 1. Expression of C4BRaf and PAK1 was confirmed by probing with anti-FLAG or anti-Myc antibodies, respectively. B, LNCaP cells were transfected with EGFP mixed with an empty vector, C4B-Raf, PAK1(K229R), or both C4B-Raf and PAK1(K229R). After 20 h, cells were serum-starved and treated with LY294002 and thapsigargin (LY + T) followed by EGF where indicated. The apoptotic cell death in GFP-positive cells was assessed by time lapse video recording. At least 250 cells in randomly chosen fields were counted for each treatment. The bars show the percentage of apoptosis 6 h after treatments; error bars show S.D. between apoptosis in individual fields. The difference between apoptosis in cells treated with and without EGF is statistically significant (p < 0.01) in all transfections except transfection with C4B-Raf and DN-PAK1 (p > 0.15). Data for multiple time points are shown in Fig. S8. C, LNCaP cells were transfected with pGDB and either with an empty vector, C4B-Raf, PAK1(K229R), or both C4B-Raf and PAK1(K229R). After 20 h, cells were serum-starved and treated as in B. 6 h after treatments, cells were lysed, and cleavage of GDB protein was assessed by probing lysates with anti-GFP antibodies. Equal loading was verified by β-actin antibodies.
Rac can be activated downstream from PI3K or independently from PI3K by receptor tyrosine kinases via the Grb2 adapter that recruits the Rac guanine nucleotide exchange factor Sos (57). Our results suggest that the latter scenario of Rac/PAK activation is realized in prostate cancer cells. Inhibition of EGF-induced BAD phosphorylation at Ser136 by N17Ras (26) is consistent with involvement of Sos in EGF-induced BAD phosphorylation at Ser136.

In LNCaP and C4-2 cells with active PI3K signaling, BAD phosphorylation at either Ser112 or Ser136 is insensitive to the expression of C4B-Raf as well as inhibition of MEK1, PAK, p70S6 kinase (Figs. 3, 4, 6, and S4), or protein kinase A (26), suggesting that Akt or other kinases are responsible for the phosphorylation of these sites. The role of Akt in phosphorylation of BAD at Ser136 has been established, but it is less clear whether Akt phosphorylates Ser112 as well (10, 52, 58). In human breast cancer cell lines and mouse breast epithelial cells, EGF induces BAD phosphorylation only at Ser112, whereas PI3K-dependent signaling controls Ser136 (21, 25).

Our data reveal the complex organization of signaling pathways that phosphorylate BAD in prostate cells. EGF as well as PI3K transmit signals to both Ser112 and Ser136. EGF-induced phosphorylation of BAD at Ser112 and Ser136 has been reported in HEK 293 cells (59), and constitutive BAD phosphorylation at Ser112 and Ser136 was observed in breast cancer cells with inactive PTEN (60). Expression of PTEN in LNCaP cells also leads to dephosphorylation of BAD at both Ser112 and Ser136 (Fig. S2). In fact, PI3K-dependent phosphorylation of BAD at Ser112 is not limited to tissue culture cells with inactive PTEN but can be also seen in mice with prostate-restricted PTEN deletion. It would not be surprising if signaling pathways connecting PI3K and BAD phosphorylation in prostate cells show redundancy and complexity comparable with signaling pathways activated by EGF.

Our results as well as other reports on BAD phosphorylation in tissue culture models reveal an intricate network of signaling pathways that control BAD phosphorylation. What is the significance of BAD phosphorylation in vivo? BAD knock-out mice show a reduction of life span due to increased death from malignancies (mostly lymphomas) and a decrease in apoptotic responses to cytotoxic stimuli but lack any developmental defects (61). Mice in which endogenous BAD is replaced with a BAD3SA construct with mutations in phosphorylation sites also develop normally but show defects in pro-B and pro-T cell survival; embryonic fibroblasts display defects in the protection from apoptosis by survival factors (62). Relatively mild effects of BAD deficiency or expression of phosphorylation-deficient BAD on mouse development are probably due to the functional redundancy among Bcl-2 homology domain 3 (BH3)-only proteins and relatively low BAD expression levels in normal tissues. In tumors, however, the role of BAD may be more prominent. Thus, loss of PTEN activity creates permissive conditions for BAD expression, and prostate tumors were shown to increase BAD levels (63). This increase is probably due to proliferative advantages that phosphorylated BAD gives to cancer cells. Indeed, involvement of BAD in stimulating survival, glycolysis, and progression through the cell cycle was recently reported (64–67).

Co-expression of EGFR and transforming growth factor-α has been detected in prostate cancer samples, and there are also reports on increased phosphorylation of ERK in primary and metastatic prostate cancer (14, 15, 68). Thus, chronic signaling through the EGFR/Ras/Raf/MEK cascade may provide an additional mechanism (besides the PI3K/Akt module) to control BAD phosphorylation in prostate cancer cells. Another mechanism that connects activation of EGFR with BAD is Rac/PAK signaling. Activation of PAK by EGF may also explain increased motility and invasion of prostate cancer cells stimulated by growth factors of the EGF family (69).

The remarkable redundancy of signaling pathways that control BAD phosphorylation in prostate cancer cells argues for the importance of keeping BAD under control. It would be of interest to test whether BAD dephosphorylation leads to involution of prostate cancer xenografts or sensitizes tumors to cytotoxic treatments. If so, kinases that phosphorylate BAD would be plausible targets for anti-cancer therapy, and analysis of BAD phosphorylation may be used as a predictor of its success.

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