

Diverse Antiapoptotic Signaling Pathways Activated by Vasoactive Intestinal Polypeptide, Epidermal Growth Factor, and Phosphatidylinositol 3-Kinase in Prostate Cancer Cells Converge on BAD^{*§}

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It has been demonstrated that vasoactive intestinal polypeptide, epidermal growth factor, and chronic activation of phosphatidylinositol 3-kinase can protect prostate cancer cells from apoptosis; however, the signaling pathways that they use and molecules that they target are unknown. We report that vasoactive intestinal polypeptide, epidermal growth factor, and phosphatidylinositol 3-kinase activate independent signaling pathways that phosphorylate the proapoptotic protein BAD. Vasoactive intestinal polypeptide operated via protein kinase A, epidermal growth factor required Ras activity, and effects of phosphatidylinositol 3-kinase were predominantly mediated by Akt. BAD phosphorylation was critical for the antiapoptotic effects of each signaling pathway. None of these survival signals was able to rescue cells that express BAD with mutations in phosphorylation sites, whereas knockdown of BAD expression with small hairpin RNA rendered cells insensitive to apoptosis. Taken together, these results identify BAD as a convergence point of several antiapoptotic signaling pathways in prostate cells.

The most effective systemic treatment for advanced prostate carcinoma is androgen ablation, which eliminates androgen-dependent prostate epithelial cells by inducing apoptosis (1, 2). However, this treatment rarely cures patients because prostate cancer cells can engage antiapoptotic mechanisms that allow them to survive without androgen. Therefore, resistance to apoptosis has been connected to prostate cancer progression (3–6).

One of the first antiapoptotic pathways to be described was signaling via the PI3K²/Akt module (7–9). The constitutive

activation of PI3K signaling due to the loss of the lipid phosphatase PTEN has been observed in various cancers, including advanced prostate cancer (10, 11). Akt1 has been identified as the most prominent kinase downstream of PI3K. It inhibits apoptosis by phosphorylation of the proapoptotic protein BAD; transcription factors of cAMP-responsive element-binding protein, YAP, and FOXO families; and by affecting glucose metabolism (12, 13). Other targets of Akt connected with regulation of apoptosis include the I κ B, Mdm2, Par-4, GSK3, and mTOR kinases (14, 15).

In addition to the constitutive activation of PI3K signaling in PTEN-deficient cells, growth factors, chemokines, and neuropeptides can protect prostate cancer cells from apoptosis via PI3K-dependent (16–19) as well as PI3K-independent mechanisms (20). For example, EGF and heregulin protect prostate cancer cells from apoptosis in the presence of PI3K inhibitors that block Akt activity (21). Expression of receptor tyrosine kinases of HER family, HER1/EGFR and HER2/neu, has been documented in prostate epithelial cells and prostate tumors. Increased levels of the EGFR ligand transforming growth factor- α has been observed in advanced prostate cancer (22–25). Thus, antiapoptotic signaling via receptor tyrosine kinases of the HER family is likely to contribute to the prostate cancer progression.

Another signaling mechanism that can regulate apoptosis is activation of G protein-coupled receptors (GPCRs). GPCR agonists, such as endothelin-1, bombesin, calcitonin, and vasoactive intestinal polypeptide (VIP), have been shown to support androgen-independent growth of prostate cancer cells (26, 27). Elevated expression of VIP and VIP receptors has been found in prostate carcinoma, and antagonists of VIP inhibit the growth of prostate cancer xenografts (28, 29). VIP is secreted by autonomic nerves in the prostate gland and also can be produced by prostate cancer cells (27, 30–32). VIP-binding sites are identified in specimens of primary prostate cancer and in PC3 and LNCaP cells derived from prostate cancer metastases (27, 33, 34). VIP has been shown to protect several cell types, including thymocytes, ovarian and prostate cancer cells from apoptosis

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² The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; VIP, vasoactive intestinal polypeptide; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; MEK, mitogen-activated protein

kinase/extracellular signal-regulated kinase kinase; PAK, p21-activated kinase; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PKA, protein kinase A; siRNA, small interfering RNA; shRNA, small hairpin RNA; GFP, green fluorescent protein; HA, hemagglutinin; LY, LY294002; BH3, Bcl homology domain 3.

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(27, 35, 36). However, the molecular mechanism of cell survival mediated by VIP is not completely understood.

A number of reports have provided evidence of interactions between signaling pathways activated by PI3K, EGF, and GPCR agonists. For example, GPCR agonists α -thrombin and lysophosphatidic acid were shown to transactivate EGFR (37), PI3K has been implicated in EGFR and HER2 survival signaling (8, 38), and the Ras/MEK pathway was reportedly activated by VIP in prostate cells (31). The goal of this study was to examine antiapoptotic mechanisms activated by PI3K, EGF, and VIP and determine whether they utilize common or separate signaling pathways.

In this paper, we report that VIP protects prostate cancer cells from apoptosis by phosphorylating BAD via a PKA-dependent mechanism. Furthermore, we found that PI3K, EGF, and VIP activate redundant, mutually independent signaling pathways that induce phosphorylation of the proapoptotic protein BAD. Knocking down BAD expression by shRNA substantially decreases sensitivity of prostate cancer cells to apoptosis, whereas expression of BAD with mutations in phosphorylation sites induces apoptosis that cannot be inhibited by antiapoptotic signals. Taken together, these results identify BAD as a convergence point of several antiapoptotic signaling pathways in prostate cancer cells.

EXPERIMENTAL PROCEDURES

Cell Lines—The prostate cancer LNCaP and C4-2 cells were a gift from Dr. Leland Chung (Emory University, Atlanta, GA). C4-2LN17Ras cells were obtained by transfecting C4-2 cells with pGL4 mixed with Tet-on and pTRE2hygN17Ras cDNA. To obtain control C4-2L cells, empty pTRE2hyg vector was used instead of pTRE2hygN17Ras. Cells were selected in G418 and hygromycin antibiotics. The clone used in this paper expresses a sufficient amount of N17Ras to inhibit ERK activation by EGF without doxycycline induction.

Antibodies and Other Reagents—Antibodies were from the following sources. BAD, phospho-specific BAD (Ser¹¹², Ser¹³⁶, and Ser¹⁵⁵), phospho-ERK (Thr²⁰²/Tyr²⁰⁴), Akt, phospho-Akt (Ser⁴⁷³), and phospho-GSK3 α (Ser²¹) were from Cell Signaling Technology (Beverly, MA); phospho-MEK1 (Ser²⁹⁸) antibodies were a gift from Andrew Catling (Louisiana State University, New Orleans, LA); anti-FLAG M2 monoclonal antibodies were from Sigma; secondary horseradish peroxidase-conjugated antibodies used for Western blots were from Amersham Biosciences. Protein G-agarose beads, DEVD-7-amino-4-methylcoumarin, VIP, and rapamycin were from Calbiochem. Recombinant EGF was from Upstate Biotechnology, Inc. (Lake Placid, NY). Scramble and Akt-siRNA were from Cell Signaling Technology (catalog numbers 6201 and 6211). All other chemicals and reagents (unless specified) were purchased from Sigma. Tissue culture reagents were purchased from Gibco.

Plasmid and DNA Constructs—pcDNA3-HA-BAD, pcDNA3-HA-BADS112A, and pcDNA3-HA-BADS112/136A constructs were from Michael Greenberg (Harvard Medical School, Boston, MA); pRK5-Myc-PAK1(K299R) and pCMV-HA-MEK1 were from Andrew Catling. EGFP was from Clontech. PKI-GFP was from Dr. Raymond Penn (Wake Forest University, Winston-Salem, NC). Dominant negative Akt (AAA-Akt) and con-

stitutively active Akt (Myr-Akt) were from Dr. Dario Alessi (University of Dundee, Scotland). The UBC-GFP construct that expresses luciferase fused to green fluorescent protein under the ubiquitin promoter was a gift from Dr. Purnima Dubey (Wake Forest University, Winston-Salem, NC).

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 at 60–70% confluence using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. The following amounts of DNA were used per 10-cm plate: 0.6 μ g of EGFP, 0.5 μ g of BAD, 1 μ g of Bcl-X_L, and 4 μ g of DN-N17Ras, PKI-GFP, DN-PAK1, AAA-Akt, or Myr-Akt expression constructs.

siRNA Experiments—For Akt-siRNA experiments, cells were cotransfected with 0.2 μ g of HA-BAD, 0.4 μ g of Bcl-X_L, and 50 nM Akt-siRNA or scramble siRNA using Metafectene transfection reagent (Biontex, Germany). For the first 24 h after transfection, cells were maintained in serum-supplemented medium and under serum-starved conditions for the following 48 h. Then cells were lysed and probed for Akt and BAD phosphorylation. For shRNA BAD knockdown experiments, a lentiviral vector (pLL3.7) was used with an shRNA insert of annealed oligonucleotides. The BAD DNA target sequences used were 5'-TGAAGGGACTTCCTCGCCCGT-3' and 5'-GGCTTG-GTCCCATCGGAAG-3'. HEK 293 cells were transfected with pLL3.7 vector containing either of these sequences or a scrambled sequence 5'-GGTACGGTCAGGCAGCTTCT-3' in combination with packaging vectors (VSVG, RSV-REV, and pMDLg/p RRE). After 48 h, supernatants were collected from these cells and used to infect LNCaP or C4-2 cells. Forty-eight hours after infection, cells were plated for subsequent experiments.

Immunoprecipitation—Twenty hours after transfection with BAD expression constructs, cells were deprived of serum for 3 h, and different treatments were given at this point as described in the figure legends. 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 μ g each of leupeptin, pepstatin, and aprotinin, 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 \times g for 10 min at 4 °C. Cell extracts were incubated with 6–8 μ g of anti-HA antibodies (12CA5) overnight at 4 °C and protein G-agarose 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 blot analysis.

Apoptosis Assays—Apoptosis was induced as follows. Cells were serum-starved overnight (16 h) and treated with 50 μ M LY294002 and 1 μ M thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum calcium ATPase. Thapsigargin alone does not induce apoptosis in prostate cancer cells within 24 h (39, 40), but in the experimental conditions that we used it synchronized caspase activation in cells treated with LY294002. This protocol shortened the time and increased the reproducibility of caspase assays and also permitted analysis of apoptosis by time lapse video recording. Apoptosis in whole cell popula-

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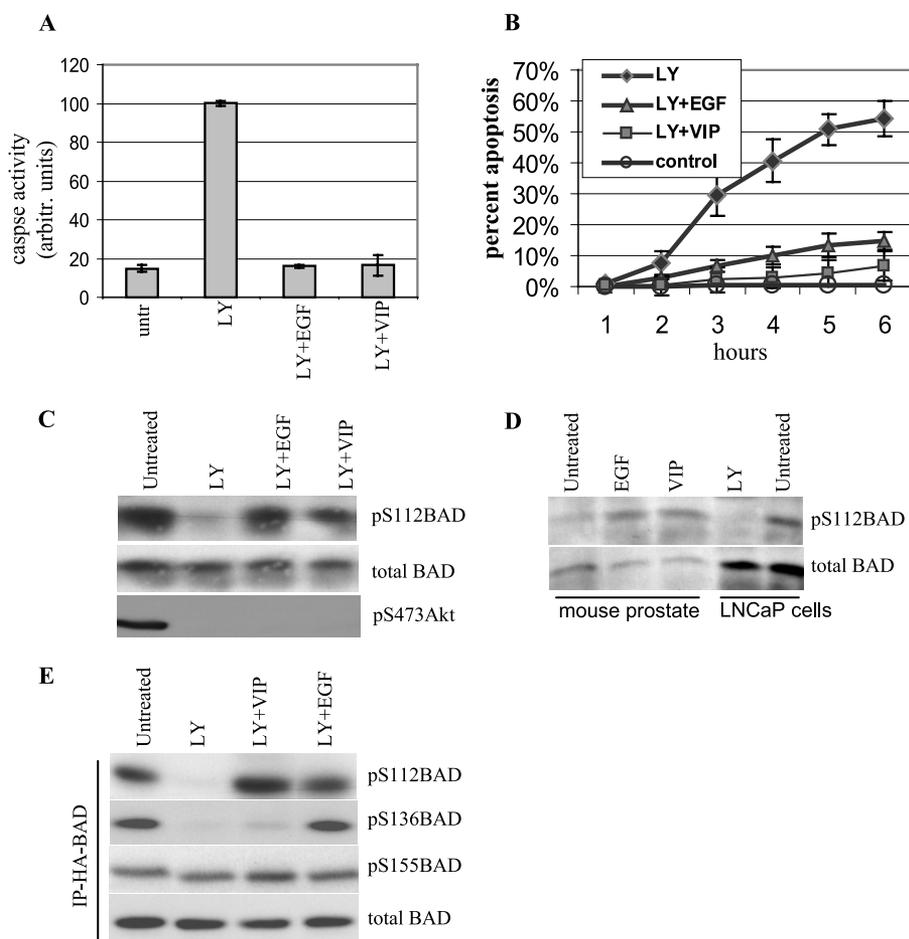


FIGURE 1. VIP and EGF protect prostate cancer cells from apoptosis and induce phosphorylation of BAD. *A*, LNCaP cells were treated with 50 μ M LY and 1 μ M thapsigargin followed by either 5 nM EGF or 100 nM VIP as indicated. After 6 h, cells were collected, lysed, and caspase activity was measured with the fluorogenic substrate Ac-DEVD-7-amido-4-trifluoromethylcoumarin (shown in relative units). *Bars* show the average of duplicate samples. *B*, cells were treated as in *A*. Apoptotic cell death (cytoplasmic blebbing and fragmentation) was followed by time lapse video recording. The percentage of apoptotic cells was calculated each hour for 6 h after treatments. Data are presented as the average of three randomly chosen fields with \sim 100 cells each. *Diamonds*, LY294002; *triangles*, LY294002 + EGF; *squares*, LY294002 + VIP; *circles*, control. *C*, phosphorylation of endogenous BAD is induced by VIP and EGF. LNCaP and C4-2L cells were treated with 50 μ M LY for 2 h followed by 5 nM EGF or 100 nM VIP for 1 h where indicated. Lysates were immunoblotted for pS473 Akt, pS112BAD, and total BAD (control for equal loading). *D*, anterior prostates were injected with 10 nM EGF, 100 nM VIP, or vehicle (10 μ l of saline). Twenty minutes after injections, the anterior prostates were removed and frozen in liquid nitrogen. Lysates of prostates and lysates of intact and LY294002-treated LNCaP cells were immunoblotted for pS112BAD and total BAD. *E*, LNCaP cells were transfected with HA-BADwt and FLAG-Bcl-X_L. Bcl-X_L was included to increase recovery of HA-BADwt from cells treated with LY294002. Twenty hours after transfection, cells were serum-starved for 3 h and treated with LY294002 for 2 h, followed by the addition of 5 nM EGF or 100 nM VIP for 1 h. HA-BAD was immunoprecipitated (IP) using 12CA5 antibodies and immunoblotted with antibodies to pS112, pS136, and pS155. Anti-BAD antibodies were used to confirm equal loading.

tions was quantified by measuring caspase-3 activity with the fluorogenic substrate Ac-DEVD-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 μ g/ml aprotinin, leupeptin, and pepstatin). Fluorescence was recorded each 15 min for 1 h, and caspase activity was expressed in arbitrary units. Apoptosis 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 (assessed as cytoplasmic blebbing and fragmentation). Apoptosis was confirmed by indirect immunofluorescence with antibodies that recognize active

caspase 3. For these experiments, cells were plated in 6-well plates and transfected by Lipofectamine with a mixture of cDNA that included GFP in a 1:9 ratio. At least four randomly chosen fields (containing on average 200–300 cells for each treatment) were recorded. Video recording was performed on an Axiovert100 microscope (Carl Zeiss, Germany) equipped with a moving stage and climate control chamber (37 $^{\circ}$ C, 5% CO₂) and controlled by Openlab software (Improvision Inc., Lexington, MA). The results reported herein were confirmed by at least two independent experiments.

Luciferase Assays—Cells were co-transfected with 0.5 μ g/ml HA-BADwt or HA-BADS112A or HA-BAD S112/136A (BAD2SA) and 0.1 μ g/ml of luciferase expressing vector UBC-GFP-luc. Twenty-four hours after transfection, cells were serum-starved for 3 h and treated with 20 μ M LY294002 and 3 μ g/ml cycloheximide to prevent changes in expression of transfected proteins by growth factors. Ten minutes later, cells were treated with either 5 nM EGF or 100 nM VIP. Six hours later, cells were washed with PBS three times and lysed, and luciferase activity was measured on a microplate luminometer according to the manufacturer's instructions using a kit from Promega (catalog number E1500).

BAD Phosphorylation in Mouse Prostate—C57BL/6 male mice were anesthetized with ketamine/xylazine mixture, the abdomen was opened, and both lobes of anterior

prostates were injected in three locations with 10 μ l of saline alone or with 10 nM EGF or with 100 nM VIP. The abdomen was then closed with a metal clip. Twenty minutes later, the anesthetized mice were euthanized by cervical dislocation, and the anterior prostates were removed and frozen in liquid nitrogen. Prostates were lysed, and phosphorylation of endogenous BAD was analyzed by immunoblotting.

RESULTS

PI3K, EGF, and VIP Protect Cells from Apoptosis and Induce BAD Phosphorylation—PI3K signaling is constitutively up-regulated in LNCaP prostate cancer cells due to a frameshift mutation of the lipid phosphatase PTEN (41). As a result, these cells can survive in serum-free medium in the absence of trophic

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factors. Recently, we have shown that inhibition of PI3K with LY294002 in LNCaP cells induces apoptosis, characterized by release of cytochrome *c* from mitochondria, activation of caspases 3 and 7, and fragmentation of the nucleus and cytoplasm. We also have demonstrated that treatment with EGF can protect LNCaP cells by inhibiting proapoptotic signaling upstream of mitochondria independent of PI3K activity and new protein synthesis (20). To test whether PI3K-independent survival can be induced by other extracellular ligands, we examined the effects of VIP on apoptosis in prostate cancer cells. Analysis of caspase activity showed that in the presence of LY294002, VIP provided protection from apoptosis comparable with that obtained with EGF (Fig. 1A). Similar results were obtained when apoptosis was measured by time lapse video recording that permits dynamic documentation of apoptosis in individual cells (Fig. 1B).

Previously, BAD, a BH3-only member of the Bcl-2 family, has been identified as a target of the PI3K/Akt antiapoptotic signaling pathway (42, 43). BAD phosphorylation at Ser¹¹² and Ser¹³⁶ facilitates BAD association with 14-3-3 proteins and inhibits apoptosis (44). We examined the status of BAD phosphorylation in prostate cancer cells treated with LY294002 alone and in combination with EGF and VIP. In LNCaP cells maintained in serum-free medium, phosphorylation of endogenous BAD was recognized with phospho-Ser¹¹²-specific antibodies. Upon treatment with LY294002, BAD became dephosphorylated; however, treatment with either EGF or VIP restored BAD phosphorylation in the absence of Akt activation (Fig. 1C). Similar effects of EGF and VIP on BAD phosphorylation and protection from apoptosis were observed in C4-2 prostate cancer cells, which exhibit more aggressive tumor growth *in vivo* (not shown). Furthermore, BAD phosphorylation at Ser¹¹² was observed in mouse prostate glands injected with EGF and VIP (Fig. 1D). The latter result demonstrates that the ability of EGF and VIP to induce phosphorylation of BAD was not limited to prostate cancer cell lines maintained in tissue culture but also occurred in the context of the intact prostate gland.

In addition to Ser¹¹², BAD can be phosphorylated at Ser¹³⁶ and Ser¹⁵⁵. Because phosphospecific antibodies against Ser¹³⁶ and Ser¹⁵⁵ are not sensitive enough to detect phosphorylation of endogenous BAD, analysis of BAD phosphorylation at these sites was performed on immunoprecipitates of HA-BAD ectopically expressed in LNCaP cells. In cells growing in serum-free conditions, HA-BAD was constitutively phosphorylated at Ser¹¹² and also at Ser¹³⁶ and Ser¹⁵⁵. Treatment with LY294002 resulted in dephosphorylation of Ser¹¹² and Ser¹³⁶ within 2 h but did not reduce Ser¹⁵⁵ phosphorylation (supplemental Fig. 1). VIP induced only Ser¹¹² phosphorylation, whereas EGF restored BAD phosphorylation at both Ser¹¹² and Ser¹³⁶ (Fig. 1E). Thus, the antiapoptotic effects of PI3K, EGF, and VIP correlate with BAD phosphorylation status.

BAD Phosphorylation Is Necessary for Cell Survival Mediated by PI3K, VIP, and EGF—To examine the role of BAD in apoptosis of prostate cancer cells, we engineered lentiviral vectors that express BAD-specific shRNA or scrambled shRNA. Infection with lentivirus that expresses BAD shRNA dramatically reduced expression of endogenous BAD (Fig. 2A). Time lapse video recording demonstrated reduced apoptosis in LNCaP

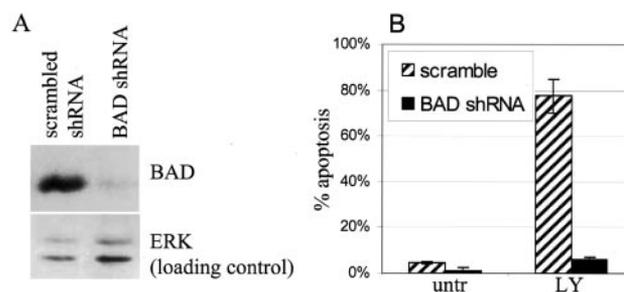


FIGURE 2. BAD expression is necessary for apoptosis. A, inhibition of endogenous BAD expression by shRNA. Lysates of LNCaP cells infected with pLL3.7 vector containing either BAD shRNA or scrambled shRNA were prepared 48 h postinfection and immunoblotted with antibodies against BAD. Equal protein loading was confirmed by antibodies against ERK. B, BAD expression is necessary for induction of apoptosis. Forty-eight hours after infection with pLL3.7 vector that carries either scrambled shRNA (striped bars) or BADshRNA (solid bars), cells were treated with 1 μ M thapsigargin and 50 μ M LY or vehicle (Me₂SO). Apoptosis was followed by time lapse video recording of GFP-positive cells. The percentage of apoptosis in 6 h after treatments was calculated. Data are presented as average of three randomly chosen fields with ~100 cells each.

and C4-2 cells where BAD expression is knocked down (Fig. 2B). Thus, BAD expression is required for apoptosis induction in prostate cancer cells.

To address the role of BAD phosphorylation in antiapoptotic signaling, we tested the survival effects of EGF and VIP in cells that express BAD with mutated phosphorylation sites (Fig. 3A). LNCaP cells were transfected with either wild type BAD (BADwt), BADA112A (where serine 112 is replaced with alanine), or BAD2SA (where both Ser¹¹² and Ser¹³⁶ are mutated). Cell survival was judged by measuring luciferase activity in cells co-transfected with luciferase expression vector and BAD constructs. A similar method utilizing a β -galactosidase reporter construct has been previously used to examine survival in cells that transiently express BAD with mutations in phosphorylation sites (45). Both EGF and VIP were able to restore survival in LY294002-treated cells that express BADwt. However, in cells that express BADA112A, the survival effect of VIP was diminished, whereas EGF still showed a strong survival effect. In cells transfected with BAD2SA, survival was lower even without LY294002 treatment than in LY294002-treated cells transfected with the other BAD constructs. Neither VIP nor EGF increased survival in cells transfected with BAD2SA. However, co-transfection of the antiapoptotic protein Bcl-X_L with BAD2SA restored survival, which is consistent with the idea that BAD2SA induces apoptosis (Fig. 3A). These results suggest that BAD phosphorylation at Ser¹¹² is necessary for VIP-mediated survival, whereas both Ser¹¹² and Ser¹³⁶ have to be mutated to inhibit the survival effects of PI3K and EGF.

To further corroborate the results of this experiment, we measured apoptosis in cells co-transfected with BAD constructs and GFP by time lapse video microscopy. Expression of BADwt and BADA112A was achieved at similar levels and was well tolerated by LNCaP cells (Fig. 3B). Inhibition of PI3K with LY294002 induced apoptosis in cells that expressed BAD constructs. Consistent with the results shown in Fig. 3A, VIP reduced apoptosis in cells that express BADwt but not in cells that express BADA112A, whereas EGF was able to decrease apoptosis in both BADwt and BADA112A-expressing cells (Fig. 3C and supplemental Fig. 2). Thus, phosphorylation of BAD at

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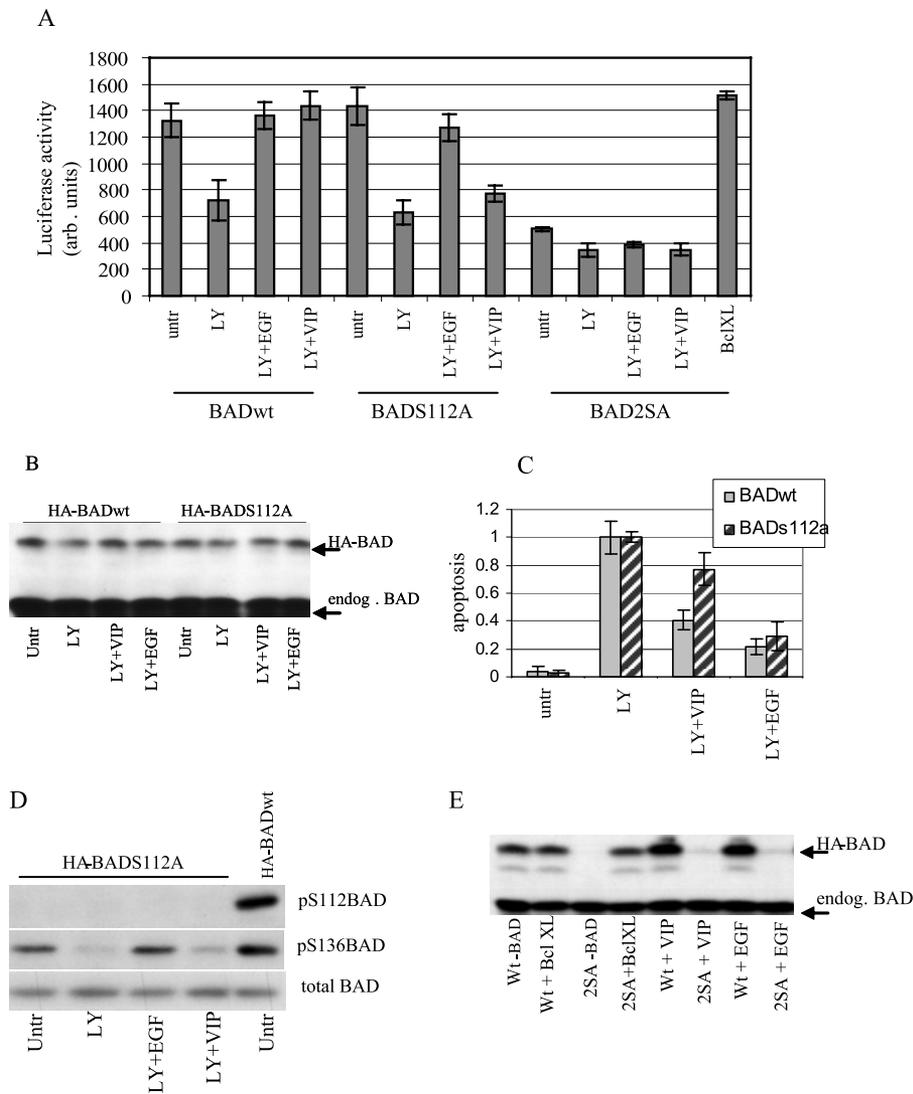


FIGURE 3. BAD phosphorylation is necessary for the antiapoptotic effects of VIP, EGF, and PI3K. *A*, cells were co-transfected with 0.5 μ g/ml of HA-BADwt or HA-BADS112A or HA-BAD S112A/S136A (BAD2SA) and 0.1 μ g/ml of luciferase expressing vector UBC-GFPuc. Twenty-four hours after transfection, cells were serum-starved for 3 h and treated with 20 μ M LY294002 and 3 μ g/ml cycloheximide to prevent changes in expression of transfected proteins by growth factors. 10 min later, cells were treated with either 5 nM EGF or 100 nM VIP. Six hours later, cells were washed three times with PBS, lysed and luciferase activity was measured. *Bars* show the average of duplicate samples. *B*, LNCaP cells were transfected with expression vectors encoding EGFP and HA-BADwt or HA-BADS112A in a 1:9 ratio. Twenty hours after transfection, cells were serum-starved for 3 h and treated with 20 μ M LY294002 followed by either 5 nM EGF or 100 nM VIP. Four hours after treatments, cells were collected, lysed, and expression of HA-BADwt and HA-BADS112A was detected by immunoblotting with anti-BAD antibodies. Endogenous BAD is shown to control equal protein loading. *C*, LNCaP cells were transfected with expression vectors encoding EGFP and HA-BADwt or HA-BADS112A in a 1:9 ratio and treated as in *B*. Apoptotic cell death in GFP-positive cells was followed by time lapse video recording. At least 250 cells from randomly chosen fields were counted for each treatment. Apoptosis in cells treated with survival agonists was expressed as fraction of apoptosis in cells treated with LY294002 (taken as 1). *Bars* represent cumulative cell death 3 h after treatments. *Solid bars*, cells transfected with HA-BADwt; *striped bars*, cells transfected with HA-BADS112A. Original time lapse results are shown in supplemental Fig. 2. *D*, LNCaP cells were transfected with expression vectors encoding FLAG-Bcl-X_L and either HA-BADS112A or HA-BADwt (1:1 ratio). Twenty hours after transfection, cells were serum-starved for 3 h and treated with 50 μ M LY294002 for 2 h, followed by the addition of 5 nM EGF or 100 nM VIP for 1 h. HA-BAD was immunoprecipitated using 12CA5 antibodies and immunoblotted with antibodies to pS112 and pS136. Anti-BAD antibodies were used to confirm equal loading. Bcl-X_L was included to increase recovery of HA-BADwt from cells treated with LY294002. *E*, HA-BAD2SA expression is not rescued by EGF, VIP, or PI3K signaling. LNCaP cells were transfected with either HA-BADwt or HA-BAD2SA mixed with pcDNA3 or Bcl-X_L (in a 1:1 ratio). Six hours after transfection, the medium was changed and supplemented with 5 nM EGF or 100 nM VIP. Twenty hours later, cells were lysed, and HA-BAD expression was analyzed by Western blotting with antibodies that recognize both endogenous (*endog.*) BAD and transfected BAD constructs.

Ser¹¹² is important for the antiapoptotic effects of VIP, whereas it is less so for EGF- or PI3K-induced survival. Apparently, phosphorylation at Ser¹³⁶ in cells with active PI3K signaling and

Ser¹³⁶ induced by constitutive PI3K signaling (compare *lanes 1* and *4* in Fig. 4A) or in BAD phosphorylation at Ser¹¹² induced by VIP in the presence of LY294002 (Fig. 4B).

in cells treated with EGF (Fig. 1E) can compensate for the lack of Ser¹¹² phosphorylation. Indeed, mutant BADS112A was phosphorylated at Ser¹³⁶ in intact cells with active PI3K signaling. Treatment with LY294002 reduced Ser¹³⁶ phosphorylation, whereas EGF restored BAD phosphorylation to the level observed in untreated cells (Fig. 3D).

In contrast to BADwt and BADS112A constructs, we were unable to express the BAD2SA construct even in cells treated with EGF or VIP. Apparently, even a small amount of BAD2SA expression was sufficient to trigger apoptosis in LNCaP cells that cannot be inhibited by PI3K, EGF, or VIP. In agreement with this idea, BAD2SA expression can be detected in cells co-transfected with antiapoptotic protein Bcl-X_L (Fig. 3E).

Taken together, our experiments with BAD knock-out by shRNA and ectopic expression of phosphorylation-deficient BAD mutants indicate that BAD phosphorylation is critical for the antiapoptotic effects of EGF, VIP, and PI3K.

Dominant Negative N17Ras Specifically Blocks EGF-induced Survival and BAD Phosphorylation—Our subsequent efforts were focused on the analysis of signaling pathways induced by PI3K, EGF, and VIP that lead to BAD phosphorylation and protection of cells from apoptosis.

Ras plays an important role in signaling by receptor tyrosine kinases, GPCRs, and PI3K (46, 47). Therefore, Ras activation can potentially contribute to the antiapoptotic effects of PI3K, EGF, and VIP. The role of Ras in BAD phosphorylation was assessed by co-transfecting dominant negative N17Ras mutant and HA-BAD in LNCaP cells. N17Ras inhibited EGF-induced BAD phosphorylation at both Ser¹¹² and Ser¹³⁶ (Fig. 4A) as well as MEK activation (Fig. 4C). However, there was no change in the status of BAD phosphorylation at Ser¹¹² and

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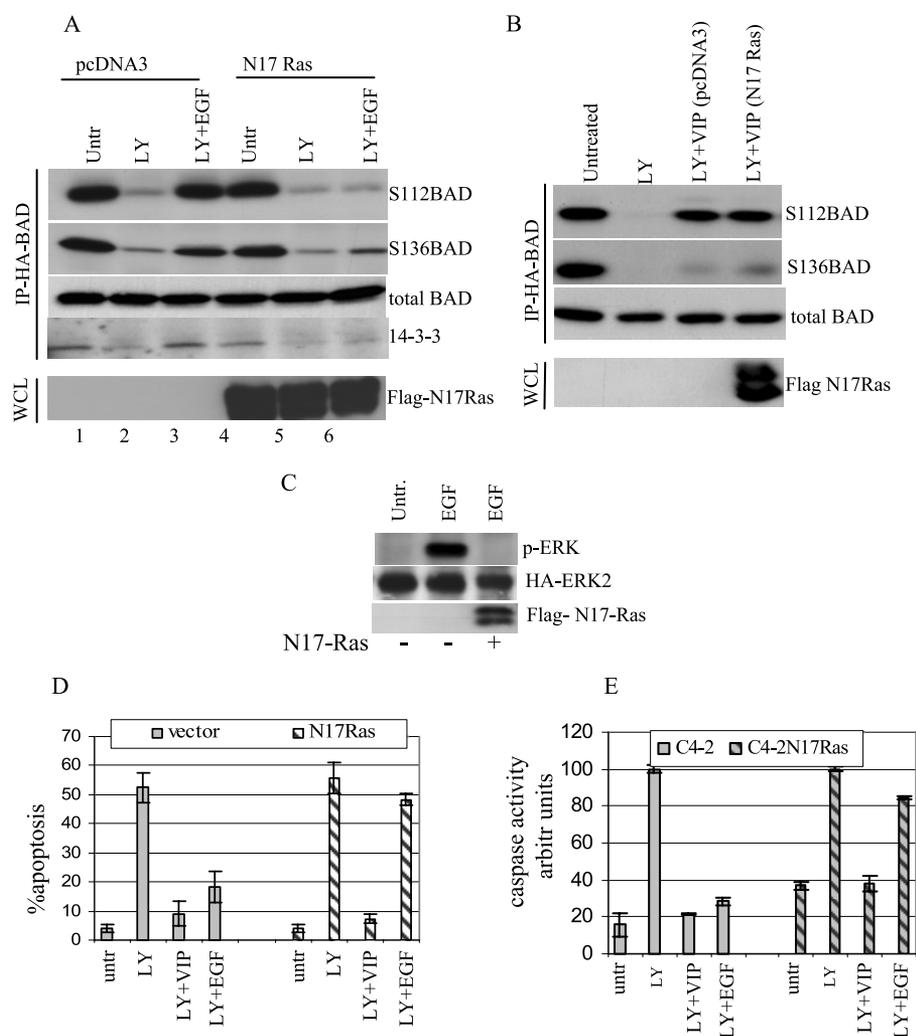


FIGURE 4. N17 Ras specifically inhibits EGF-induced BAD phosphorylation and survival. LNCaP cells were co-transfected with expression vectors that encode HA-BADwt, FLAG-Bcl-X_L, and either empty pcDNA3 vector or FLAG-N17Ras. Twenty hours after transfection, cells were serum-starved for 3 h, incubated with 50 μ M LY294002 for 2 h, and then treated with either 5 nM EGF (A) or 100 nM VIP (B) for an additional 1 h. HA-BAD was immunoprecipitated (IP), and phosphorylation of BAD was detected as in Fig. 3D. Immunoprecipitates were also immunoblotted with antibodies to 14-3-3 protein. Expression of N17Ras was verified by probing cell lysates (WCL) with anti-FLAG antibodies. C, dominant negative N17Ras inhibits MAPK activity in LNCaP cells. LNCaP cells were transfected with HA-ERK and either empty vector or FLAG-N17Ras. Twenty hours after transfection, cells were serum-starved for 3 h and treated with 30 ng/ml EGF for 15 min. HA-ERK was immunoprecipitated with 12CA5 antibodies, and phosphorylation of ERK was detected using phospho-ERK (Thr²⁰²/Tyr²⁰⁴) antibodies. Equal loading was verified by anti-HA antibodies. Expression of DN-Ras was confirmed by anti-FLAG antibodies. D, dominant negative N17Ras specifically inhibits EGF-induced cell survival. LNCaP cells were co-transfected with EGFP and either empty pcDNA3 vector or N17Ras (1:9 ratio). After 20 h, cells were serum-starved and treated as in Fig. 1A to induce apoptosis. Six hours later, the percentage of apoptosis was determined by counting cells with fragmented cytoplasm among GFP-positive cells. *Solid bars*, pcDNA3 vector; *striped bars*, N17Ras. At least 300 cells were counted for each treatment. The *error bars* show deviation of individual fields from the average. E, C4-2L cells (*solid bars*) and C4-2LN17Ras cells (*striped bars*) that stably express N17Ras were serum-starved for 20 h and apoptosis was induced as in Fig. 1A. Six hours later, cells were collected and lysed, and caspase activity was measured with the fluorogenic substrate Ac-DEVD-7-amido-4-trifluoromethylcoumarin.

Since phosphorylation of BAD at Ser¹¹² and Ser¹³⁶ results in its association with 14-3-3, we examined how inhibition of Ras signaling affects this protein complex. In untreated cells, phosphorylated BADwt was co-precipitated with 14-3-3. This interaction and BAD phosphorylation was markedly reduced by LY294002 treatment (Fig. 4A, lanes 1 and 2). EGF restored the binding of BAD to 14-3-3 in vector-transfected LNCaP cells but failed to do so in cells expressing N17Ras (Fig. 4A, lanes 3 and 6).

Next, we studied the effect of N17Ras on cell survival. Transient transfection of N17Ras into LNCaP cells completely

inhibited the cytoprotective effect of EGF in LY294002-treated cells; however, it did not interfere with antiapoptotic effects of VIP, nor did it induce apoptosis in cells with active PI3K signaling (Fig. 4D). Inhibition of EGF-dependent survival was also observed in experiments with C4-2LN17Ras cells that stably express N17Ras, where caspase activity was used to measure apoptosis (Fig. 4E). Together, these results indicate that Ras activity is necessary for EGF-induced BAD phosphorylation and protection from apoptosis. In contrast, VIP and PI3K use Ras-independent mechanisms to regulate BAD phosphorylation and cell survival.

Protein Kinase A Mediates VIP-induced BAD Phosphorylation and Survival—In prostate cells, VIP exerts its biological effects through the VPAC1 receptor, a member of the GPCR superfamily. Upon binding to VPAC1, VIP activates adenylyl cyclase and increases cAMP levels (33). In turn, cAMP activates PKA, which has been shown to phosphorylate BAD and thereby protect cells from apoptosis (48).

To address the role of PKA in BAD phosphorylation and cell survival induced by PI3K, VIP, and EGF, we used an inhibitory molecule, PKI (49), expressed as a chimera with GFP (PKI-GFP) (50). Co-transfection of LNCaP cells with PKI-GFP and BADwt significantly inhibited VIP-induced phosphorylation of BAD at Ser¹¹². However, PKI-GFP expression had no effect on the PI3K-dependent steady state level of BAD phosphorylation or BAD phosphorylation induced by EGF (Fig. 5A).

Since PKI-GFP inhibited VIP-induced BAD phosphorylation at Ser¹¹², we examined its effect on the association of 14-3-3 with BAD. VIP restored the binding of BAD to 14-3-3 protein in cells transfected with GFP but failed to do so in cells transfected with PKI-GFP. However, expression of PKI-GFP did not decrease the interaction between 14-3-3 and BAD in cells treated with EGF or in cells with active PI3K signaling (Fig. 5A). Thus, our experiments with PKI-GFP show that BAD phosphorylation and interaction with 14-3-3 induced by VIP depends on PKA activity, whereas PI3K and EGF induced BAD phosphorylation via PKA-independent mechanisms.

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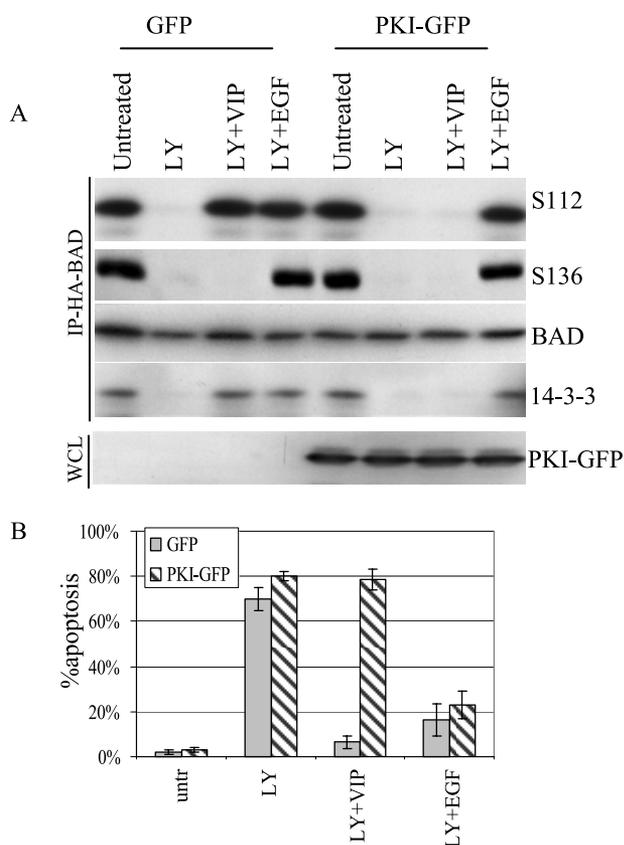


FIGURE 5. PKI selectively inhibits phosphorylation of BAD and survival induced by VIP. *A*, PKI-GFP inhibits VIP-induced BAD phosphorylation. LNCaP cells were co-transfected with expression vectors that encode HA-BADwt, Bcl-X_L, and either GFP or PKI-GFP. Twenty hours after transfection, cells were serum-starved for 3 h, incubated with LY294002 for 2 h, and then treated with either EGF or VIP for 1 h. Phosphorylation of immunoprecipitated (IP) HA-BAD was detected as in Fig. 3D. Immunoprecipitates were also immunoblotted with antibodies to 14-3-3 protein. Expression of PKI-GFP was verified with GFP-specific antibodies. *B*, VIP-induced survival is inhibited by PKI-GFP. LNCaP cells were transfected with either PKI-GFP or empty vector and GFP. Twenty hours after transfection, cells were serum-starved and treated as in Fig. 1A. Apoptosis was followed by time lapse video recording. The bars show percentage of apoptosis in GFP-positive cells 6 h after treatments. Solid bars, GFP; striped bars, PKI-GFP. At least 250 cells were counted for each treatment. Error bars, deviation of individual fields from the average.

Next, we studied the effect of PKI-GFP on cell survival mediated by VIP, EGF, and PI3K. Apoptosis in cells that express GFP or PKI-GFP was determined by time lapse video recording of GFP-positive cells followed by counting the percentage of cells with apoptotic morphology. As shown in Fig. 5B, PKI-GFP did not significantly decrease survival effects of PI3K or EGF. In contrast, the antiapoptotic effect of VIP was completely abolished in cells where PKA activity was inhibited by PKI-GFP. Thus, the PKA inhibitor PKI-GFP suppressed VIP-induced BAD phosphorylation and cell survival without altering the effects of PI3K or EGF on these parameters.

Akt Is a Principal BAD Kinase Downstream of PI3K in LNCaP and C4-2 Cells—Since PI3K-dependent BAD phosphorylation and cell survival are observed despite inhibition of Ras or PKA, we decided to address the role of PI3K effectors in further detail. The biological effects of PI3K activation are mediated by a wide array of downstream kinases. Akt, p70S6 kinase, and the PAK family of kinases are all known to be activated downstream of PI3K and phosphorylate BAD (42, 51, 52). Akt and p70S6

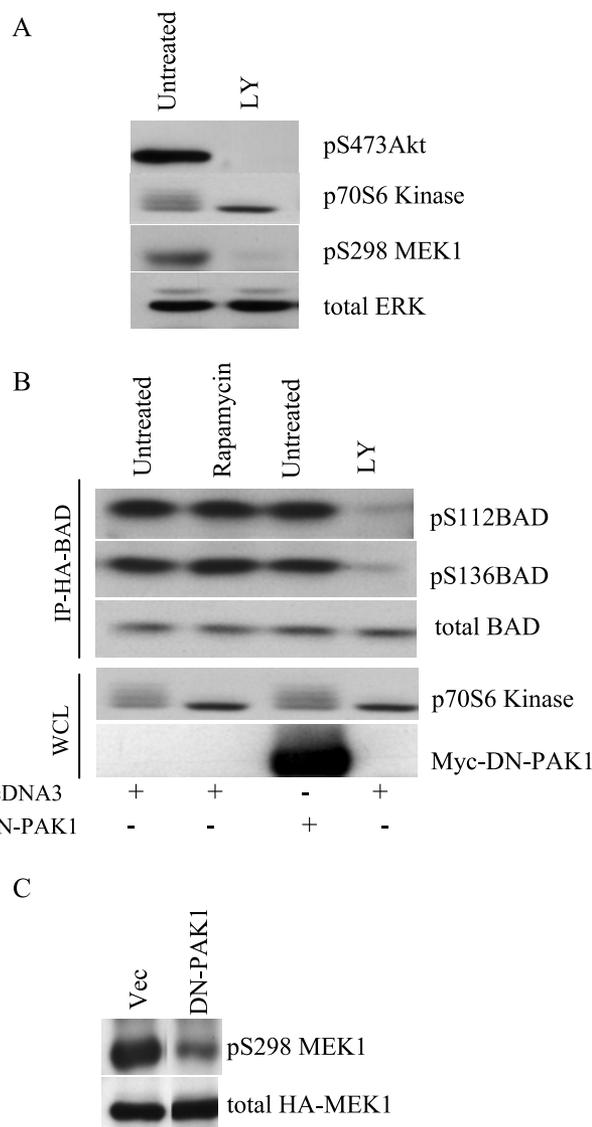


FIGURE 6. BAD phosphorylation in cells with active PI3K signaling is independent from P70S6 kinase or PAK1. *A*, Akt, p70S6K, and PAK1 are constitutively active in LNCaP cells. LNCaP cells were serum-starved for 3 h and either left untreated or treated with 50 μ M LY294002 for 3 h. Cells lysates were analyzed for Akt phosphorylation with pS473 phosphospecific antibodies and p70S6K band shift using p70S6 kinase antibodies. Detection of MEK1 phosphorylation at Ser²⁹⁸ with phosphospecific antibodies was used to monitor PAK1 activity. Equal loading was verified with antibodies against ERK. *B*, BAD phosphorylation in LNCaP cells is insensitive to rapamycin or dominant negative Myc-PAK1(K299R). LNCaP cells were transfected with expression vectors that encode HA-BAD, Bcl-X_L, and either empty pcDNA3 vector or Myc-PAK1(K299R). Twenty hours after transfection, cells were serum-starved for 3 h and treated with 10 nM rapamycin or 50 μ M LY294002 for 3 h. HA-BAD was immunoprecipitated, and phosphorylation was detected as in Fig. 3D. Cell lysates (WCL) were immunoblotted with antibodies to p70S6 kinase to determine inhibition of mTOR by rapamycin and with c-Myc-specific antibodies to follow expression of Myc-PAK1(K299R). *C*, dominant negative (DN) PAK1 inhibits MEK1 (Ser²⁹⁸) phosphorylation. LNCaP cells were transfected with HA-MEK1 and either empty pcDNA3 vector or DN-PAK1. Twenty hours after transfection, cells were serum-starved for 3 h and lysed. HA-MEK1 was immunoprecipitated with 12CA5 antibodies, and phosphorylation of MEK1 at Ser²⁹⁸ was detected by antibodies specific for phospho-MEK1 (S298).

kinases have been shown to phosphorylate BAD at Ser¹³⁶, whereas PAK1 phosphorylates both Ser¹¹² and Ser¹³⁶. To test whether Akt, p70S6, and PAK kinases are regulated by PI3K in LNCaP cells, the effect of LY294002 on their activation was followed.

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Activation of Akt and p70S6 kinases were assessed using antibodies that recognize their active forms, whereas PAK activation status was judged by measuring phosphorylation of Ser²⁹⁸ on MEK1, which is widely used to monitor PAK activity (53). Fig. 6A shows that Akt, p70S6, and PAK kinases are active in LNCaP cells maintained in serum-free medium, and their activity is reduced upon treatment with LY294002. To determine which of these kinases are responsible for phosphorylation of BAD, we inhibited p70S6 kinase with rapamycin and blocked PAK1 activity with DN-PAK1 (PAK1-K299R). As shown in Fig. 6B, rapamycin suppressed phosphorylation of p70S6 kinase without diminishing phosphorylation of BAD at Ser¹¹² and Ser¹³⁶. Likewise, expression of DN-PAK1 did not affect BAD phosphorylation, although it suppressed MEK1 phosphorylation at Ser²⁹⁸ and, hence, endogenous PAK activity (Fig. 6C).

To determine the role of Akt kinase in BAD phosphorylation, LNCaP cells were transfected with a dominant negative Akt-3A (where Ser⁴⁷³, Thr³⁰⁸, and Lys¹⁷⁹ are mutated to alanines). Expression of Akt-3A in LNCaP cells with active PI3K signaling reduced both Ser¹¹² and Ser¹³⁶ phosphorylation, with stronger inhibition of Ser¹³⁶ phosphorylation (Fig. 7A, lane 3). The role of Akt in BAD phosphorylation was further tested by expression of a constitutively active myristoylated Akt construct. In cells that express Myr-Akt, BAD phosphorylation was maintained at both Ser¹¹² and Ser¹³⁶ despite inhibition of PI3K by LY294002 (Fig. 7A, lane 6). In agreement with the preferential inhibition of Ser¹³⁶ by the dominant negative Akt mutant, the effect of constitutively active M-Akt on Ser¹³⁶ markedly surpassed the increase in Ser¹¹² phosphorylation.

To further clarify the role of Akt in BAD phosphorylation, we inhibited Akt expression with siRNA. As shown in Fig. 7B, co-transfection of BAD and Akt siRNA inhibited BAD phosphorylation at Ser¹³⁶ in both LNCaP and C4-2 cells by ~80%, which is comparable with inhibition by dominant negative Akt-3A. Still, Ser¹¹² phosphorylation was decreased only by 30%, which suggests that kinase(s) other than Akt contribute to BAD phosphorylation at this site. To confirm Akt inhibition, we

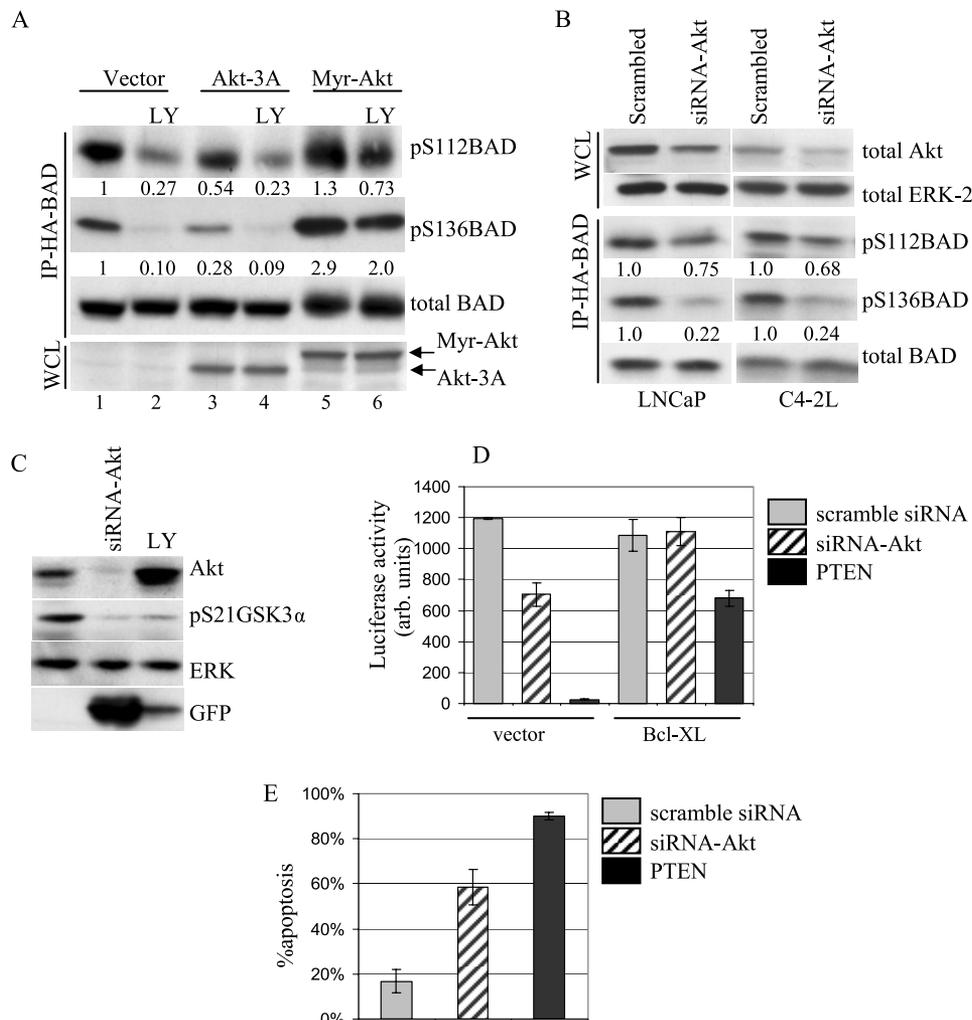


FIGURE 7. Akt mediates phosphorylation of BAD in cells with active PI3K. A, LNCaP cells were co-transfected with expression vectors encoding HA-BAD, Bcl-X_L, and either empty pcDNA3 vector, dominant negative Akt-3A, or constitutively active Myr-Akt. Twenty hours after transfection, cells were serum-starved for another 20 h and incubated with 50 μ M LY294002 for 3 h. Phosphorylation of BAD was detected with phosphospecific antibodies as in Fig. 3D. Whole cell lysates (WCL) were immunoblotted for Akt constructs. B, LNCaP or C4-2L cells were co-transfected with Bcl-X_L, HA-BADwt, and either Akt siRNA or scrambled siRNA. Twenty-four hours after transfection, cells were serum-starved for another 48 h, and whole cell lysates were probed for Akt and ERK. Phosphorylation of BAD was analyzed with phosphospecific antibodies. Signal intensity from phosphorylated BAD was normalized to total BAD levels and expressed in relative units. One relative unit is BAD phosphorylation in cells transfected with pcDNA3 vector (A) or scrambled shRNA (B). C, siRNA-Akt inhibits GSK3 phosphorylation. LNCaP cells co-transfected with GFP and siRNA-Akt were separated into GFP-positive and GFP-negative populations on a fluorescence-activated cell sorter, and expression of Akt and phosphorylation of GSK-3 was examined by immunoblotting with antibodies to Akt and phospho-GSK3 α . Lysates of unsorted cells transfected with GFP and treated with 50 μ M LY294002 are included to control inhibition of GSK3 phosphorylation. The quality of cell sorting is controlled by immunoblotting with anti-GFP antibodies; equal protein loading is verified by anti-ERK antibodies. D, siRNA-Akt inhibits cell survival. Cells were transfected with luciferase expression construct UBC-GFP-luc mixed with scrambled siRNA or siRNA-Akt or PTEN cDNA. To discriminate between the effects of PTEN and siRNA-Akt on protein synthesis and apoptosis, Bcl-X_L was included in control transfections. Luciferase activity was measured 72 h after transfections. Error bars reflect deviations from the average of duplicate wells. Similar results were obtained from two separate measurements. E, siRNA-Akt increases apoptosis. Cells were transfected with GFP mixed with scrambled siRNA or siRNA-Akt or PTEN cDNA. 24 h after transfections, cells were placed in serum-free media and GFP-positive cells were followed by time lapse video recording. At least 200 cells were counted for each transfection. Bars show the percentage of apoptosis 72 h after transfections. Error bars show S.D. between apoptosis in randomly chosen fields.

examined the effect of siRNA-Akt on the phosphorylation of endogenous GSK3 (an established Akt substrate (54)). For this purpose, cells were co-transfected with siRNA-Akt and GFP, and GFP-positive and GFP-negative cells were separated by a fluorescence-activated cell sorter. As shown in Fig. 7C, GSK3 phosphorylation in GFP-positive cells is decreased to the level observed in cells treated with LY294002.

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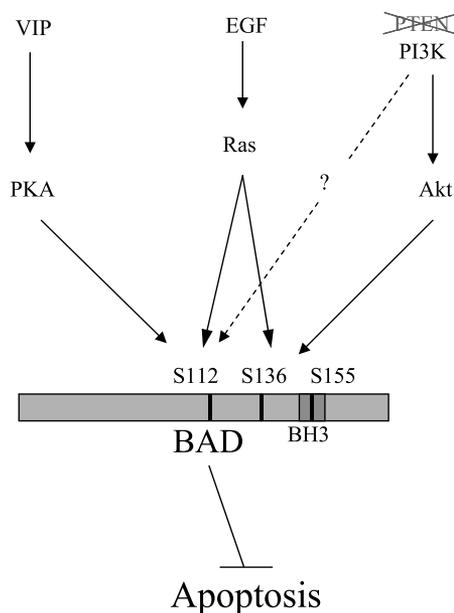


FIGURE 8. Antiapoptotic signaling pathways in prostate cancer cells converge on BAD. VIP, EGF, and PI3K induce BAD phosphorylation by independent signaling mechanisms. VIP-induced phosphorylation at Ser¹¹² depends on PKA but is not inhibited by N17Ras or the PI3K inhibitor LY294002. EGF-induced phosphorylation at Ser¹¹² and Ser¹³⁶ is inhibited by N17Ras but is independent from PKA or PI3K/Akt signaling. Constitutive PI3K signaling induces phosphorylation at Ser¹¹² and Ser¹³⁶. Phosphorylation at Ser¹³⁶ depends on Akt, whereas phosphorylation at Ser¹¹² is partially regulated via Akt and another, yet to be identified, kinase.

To address the role of Akt in apoptosis regulation in prostate cells, we examined survival in cells co-transfected with a luciferase construct (UBC-GFP_{luc}) and either siRNA-Akt or scrambled siRNA or PTEN cDNA. Transfection with siRNA-Akt decreased luciferase activity compared with cells transfected with scrambled siRNA. Because the difference in luciferase activity between siRNA-Akt and scramble siRNA transfected cells was reduced dramatically when Bcl-X_L was included in the transfection mix, we reasoned that reduction of luciferase reflects decreased survival in cells transfected with siRNA-Akt (Fig. 7D). To reinforce this point, we examined apoptosis in cells co-transfected with siRNA-Akt and GFP by time lapse video recording (Fig. 7E). Transfection with siRNA-Akt increased apoptosis within 72 h although to a lesser degree than that observed with re-expression of PTEN that has been shown to reduce BAD dephosphorylation at both Ser¹¹² and Ser¹³⁶ (55).

Taken together, our results support the role of Akt as a principal kinase that phosphorylates BAD at Ser¹³⁶ downstream of PI3K in LNCaP and C4-2 prostate cancer cells. Phosphorylation of BAD at Ser¹¹² downstream of PI3K is partially regulated by Akt and another, yet to be identified, kinase.

DISCUSSION

Results presented in this paper show that antiapoptotic signals activated by PI3K, EGF, and VIP converge on BAD, a member of the BH3-only family of proapoptotic proteins. BAD was the first proapoptotic protein shown to be inhibited by phosphorylation. In its dephosphorylated form, BAD interacts with Bcl-X_L in the outer mitochondrial membrane and increases apoptosis. Phosphorylation at Ser¹¹², Ser¹³⁶, or both sites facil-

itates interaction with 14-3-3 proteins, whereas phosphorylation of Ser¹⁵⁵ (positioned within the BH3 domain) directly inhibits binding to Bcl-X_L. In the phosphorylated states, BAD is retained in the cytoplasm by 14-3-3 and, thus, cannot bind Bcl-X_L and increase apoptosis (44).

After it was identified as a target of antiapoptotic signaling by the PI3K/Akt pathway (42, 43), BAD was shown to be phosphorylated by numerous protein kinases, including PKA, p90Rsk, p70S6, and PAK kinases (51, 52, 56, 57). Most reports describing BAD phosphorylation emphasized the dominant role of a single signaling pathway that regulates BAD phosphorylation in a given cell type. In HEK293 and MDA-468 cell lines, EGF and PI3K send nonoverlapping signals that stimulate BAD phosphorylation at Ser¹¹² and Ser¹³⁶, respectively (58, 59).

According to our data, regulation of BAD phosphorylation in prostate cells appears to be more complex. PI3K and EGF activate independent signaling mechanisms that induce phosphorylation of both Ser¹¹² and Ser¹³⁶ sites. Analysis of PI3K-dependent BAD phosphorylation shows that Ser¹³⁶ is the primary target of Akt, whereas Ser¹¹² phosphorylation is only partially Akt-dependent. This is in agreement with earlier publications that demonstrated that Ser¹³⁶ is the main Akt site (42). Partial dependence of Ser¹¹² phosphorylation on Akt was previously shown in a kinase reaction *in vitro* (60). Further experiments are needed to identify the PI3K-dependent Ser¹¹² kinase and its role in apoptosis regulation.

Our results on EGF-induced BAD phosphorylation reveal the mechanism of EGF-mediated survival in prostate cells. Extending other reports that connected the survival effect of EGF with BAD phosphorylation at Ser¹¹² (58, 59, 61), we show that both Ser¹¹² and Ser¹³⁶ are phosphorylated in a Ras-dependent but PI3K-independent fashion. The evidence from xenograft studies that supports the role of Ras in prostate cancer warrants detailed analysis of this signaling mechanism (62). Our recent experiments have shown that BAD phosphorylation induced by EGF is controlled by two parallel pathways. One pathway is mediated via Raf/MEK and another by Rac/PAK signaling.³

In addition to EGF and PI3K signaling, BAD phosphorylation at Ser¹¹² is induced by VIP. BAD phosphorylation at Ser¹¹² is sufficient for binding to 14-3-3 protein and inhibition of the proapoptotic function of BAD. Furthermore, BAD phosphorylation at Ser¹¹² is necessary for the antiapoptotic effect of VIP (Fig. 6). This effect of VIP is mediated by PKA, which is not activated by either PI3K or EGF in prostate cells (63).

In conclusion, PI3K, EGF, and VIP protect prostate cancer cells from apoptosis by independent signaling pathways that operate via Akt, Ras, and PKA and converge on BAD (Fig. 8). Thus, BAD plays the role of a signaling node that integrates diverse antiapoptotic signals in prostate cancer cells.

These results and the previous report on inactivation of BAD by serum-induced increase of Bcl-X_L expression (64) uncover a remarkable redundancy of BAD regulation in prostate cancer cells.

Why are several pathways needed to control BAD activity when a single pathway is sufficient to inactivate it (particularly

³ K. S. R. Sastry, Y. Karpova, and G. Kulik, submitted for publication.

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in cells with inactive PTEN where PI3K signaling is constitutively “on”? One possible explanation is that cells maintain a delicate balance between dephosphorylated BAD and its antiapoptotic counterparts Bcl-X_L and Bcl-2. Even a small excess of dephosphorylated BAD is sufficient to tilt this balance toward apoptosis. This idea is supported by experiments in Fig. 3E, which show that LNCaP cells can express BAD2SA with mutated phosphorylation sites only when expression of Bcl-X_L is increased as well. The high proapoptotic potential makes BAD phosphorylation a critical determinant for the survival of prostate cancer cells. Thus, “backup” signaling mechanisms may become necessary when the dominant signal from PI3K is reduced.

A possible explanation for the high proapoptotic potential of BAD in LNCaP cells is that constitutive BAD phosphorylation in cells with increased PI3K activity removes the selection pressure from other antiapoptotic mechanisms that can neutralize dephosphorylated BAD. This makes PTEN-negative prostate cancer cells especially sensitive to apoptosis induced by phosphorylation-deficient BAD. In contrast, cells with normal PI3K signaling may better tolerate expression of dephosphorylated BAD.

Further research is needed to test whether BAD phosphorylation is as important for prostate tumor development *in vivo* as it is for the survival of prostate cancer cells in tissue culture. Analysis of BAD expression and phosphorylation in tumor samples from prostate cancer patients may be a first step in that direction.

There is ample evidence that supports the involvement of signaling pathways that control BAD phosphorylation in prostate tumorigenesis. In a significant proportion of prostate tumors, PI3K/Akt signaling is constitutively elevated due to deletions or inactivating mutations of the lipid phosphatase PTEN (10). Co-expression of EGFR and its ligand, transforming growth factor- α , has been observed in tissue sections of prostate cancer biopsies (23). Consistently, prostate-specific deletion of PTEN induces prostate cancer in mice and tyrosine kinase inhibitors of HER family suppress the growth of prostate cancer xenografts (65, 66).

In addition to PI3K and EGFR signaling that were shown to control BAD phosphorylation in other tissues, we made a novel observation that VIP induces BAD phosphorylation in prostate cells. VIP is produced by neuroendocrine cells present in normal prostate and prostate tumors (30, 32). Normal prostate epithelial cells and prostate tumors express VPAC1 receptors that belong to the GPCR receptor superfamily and activate PKA (34). It is noteworthy that in advanced prostate tumors, the number of neuroendocrine cells is increased (67). Therefore, VIP/PKA signaling may play a role in maintaining BAD in a phosphorylated state in prostate cancer cells adjacent to neuroendocrine cells by a paracrine mechanism. In this manner, neuroendocrine cells may activate an alternative antiapoptotic mechanism that can support tumor growth in the presence of PI3K and EGFR inhibitors. Reports on synergism between inhibitors of PKA and EGFR kinases in prostate cells and colon tumor xenografts provide circumstantial evidence that support the significance of interaction between EGFR and PKA signaling (68, 69). It remains to be seen if BAD phosphorylation forms

the basis for this synergism, as it was recently demonstrated for inducible expression of PTEN and EGFR inhibitor in breast cancer xenografts (59).

In summary, we have identified BAD as a convergence point of diverse antiapoptotic signaling pathways and demonstrated the key role of BAD in the regulation of apoptosis in prostate cancer cells in tissue culture. Future experiments are needed to determine whether a similar complexity of BAD regulation exists in prostate tumors *in vivo*. In this case, information about the signaling network that controls BAD phosphorylation will help to select an optimal combination of kinase inhibitors, whereas analysis of BAD phosphorylation status in tumors may be used to predict treatment outcome.

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