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Protection from Rapamycin-Induced Apoptosis by Insulin-Like Growth Factor-I Is Partially Dependent on Protein Kinase C Signaling

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Abstract

Rapamycin-induced apoptosis in sarcoma cells is inhibited by insulin-like growth factor-I (IGF-I) through a signaling pathway independent of Ras-extracellular signal-regulated kinase 1/2 and Akt. IGF-I induces Bad phosphorylation (Ser^{112}, Ser^{136}, and Ser^{155}) in a pathway involving phosphoinositide 3′ kinase (PI3K) and protein kinase C (PKC; μ, ε, or θ) resulting in sequestering Bad from mitochondria and subsequently interacting with 14-3-3γ in the cytosol. Gene knockdown of Bad, Bid, Akt1, Akt2, PKC-μ, PKC-ε, or PKC-θ was achieved by transient transfection using small interfering RNAs. Results indicate that IGF-I signaling to Bad requires activation of PI3K and PKC (μ, θ, ε) but not mTOR, Ras-extracellular signal-regulated kinase 1/2, protein kinase A, or $p90^{RSK}$. Wortmannin blocked the phosphorylation of PKC-μ (Ser^{744}/Ser^{748}), suggesting that PI3K is required for the activation of PKCs. PKCs phosphorylate Bad under *in vitro* conditions, and the association of phosphorylated Bad with PKC-μ or PKC-ε, as shown by immunoprecipitation, indicated direct involvement of PKCs in Bad phosphorylation. To confirm these results, cells overexpressing pEGFP-N1, wt-Bad, or Bad with a single site mutated (Ser^{112}Ala; Ser^{136}Ala; Ser^{155}Ala), two sites mutated ($Ser^{112/136}$Ala; $Ser^{112/155}$Ala; $Ser^{136/155}$Ala), or the triple mutant were tested. IGF-I protected completely against rapamycin-induced apoptosis in cells overexpressing wt-Bad and mutants having either one or two sites of phosphorylation mutated. Knockdown of Bid using small interfering RNA showed that Bid is not required for rapamycin-induced cell death. Collectively, these data suggest that IGF-I–induced phosphorylation of Bad at multiple sites via a pathway involving PI3K and PKCs is important for protecting sarcoma cells from rapamycin-induced apoptosis. *Cancer Res; 70(5); 2000–9. ©2010 AACR.*

Introduction

The BH3-only protein Bad is unique, because its functions are tightly regulated by serine phosphorylation (1, 2). In the hypophosphorylated form, Bad interacts with either Bcl-2 or Bcl-X_L to neutralize their antiapoptotic functions, and this neutralization is believed to account for its proapoptotic functions. Inactive Bad is highly phosphorylated by survival signals and binds to 14-3-3 scaffold proteins and thus cannot interact with Bcl-2 or Bcl-X_L (2, 3). Five phosphorylation sites have been reported for Bad. Phosphorylation at Ser^{112} and Ser^{136} is involved in 14-3-3 binding (4). Published results reveal that phosphorylation of Ser^{136} is accomplished predominantly by Akt or p70S6 kinase (5, 6), whereas mitochondrially localized protein kinase A, Rsk, and Pak1 have all been shown to phosphorylate Ser^{112} (7–10). Ser^{170} is another site that is phosphorylated in cytokine-dependent cell survival (11). Recent reports indicate that Ser^{128} is phosphorylated by cdc2 during induction of apoptosis in cerebellar granular neurons. Phosphorylation of Ser^{128} has also been implicated in dissociation of Bad from 14-3-3 (12).

Recently, it has been shown that phosphorylation of Bad at Thr^{201} by JNK1 promotes glycolysis through activation of phosphofructokinase-1 (13). There is growing information in the literature that the BH3-only protein plays an essential role in cytokine deprivation–induced apoptosis in mast cells (5). BH3-only members may initiate apoptosis by directly binding to the essential cell death mediators Bax and Bak. Alternatively, they can act by engaging their prosurvival Bcl-2–like relatives (14). In one study, phosphorylation at Ser^{136} and association with 14-3-3 was found to be essential for its growth-promoting effect (15), whereas, in the other study, only association with Bcl-X_L was shown to be required, independent of the phosphorylation state of Bad (16).

Mammalian isoforms of 14-3-3 bind and modify the functions of a wide variety of critical signaling molecules. One function of 14-3-3 is to promote cell survival, as inhibition of apoptosis results from the binding of many 14-3-3 ligands,

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including Bad, ASK1, and forkhead transcription factors (17). 14-3-3 forms a very stable complex with phosphorylated Bad and plays a significant role in the regulation of Bad function.

Rapamycin, a selective inhibitor of mTORC1 signaling, causes G_1-phase accumulation and, under growth factor–deficient conditions, p53-independent apoptosis. We have shown that rapamycin-induced apoptosis is prevented by exogenous insulin-like growth factor-I (IGF-I) through a signaling pathway independent of Ras-extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt (18) and that combining rapamycin with an antibody that blocks ligand binding to IGF-IR is synergistic against most sarcoma xenograft tumor models (19). Our initial observation that overexpression of Bcl-2 significantly protects cells from rapamycin-induced apoptosis stimulated us to extend both pharmacologic and genetic studies to explore whether phosphorylation of Bad is involved in IGF-I–mediated rescue.

Materials and Methods

Inhibitors. Rapamycin, wortmannin, LY294002, PD98059, calphostin-C, chelerythrine chloride, KT5720, forskolin, and phorbol 12-myristate 13-acetate (PMA) were dissolved in DMSO before being added to culture medium (final concentration 0.1%).

Cell lines and growth conditions. The human cell lines Rh1 and Rh30 have been described (20) and were grown in antibiotic-free RPMI 1640 supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine at 37°C in an atmosphere of 5% CO_2. For serum-free experiments, cells were cultured in modified MN2E medium as described previously (20).

ApoAlert assay. We used the ApoAlert Annexin V-FITC Apoptosis kit (Clontech) as described previously (20).

Western blot analysis. Immunoblotting methods were as described previously (20) with minor modifications. The secondary antibody was either horseradish peroxidase–conjugated goat anti-rabbit IgG or horseradish peroxidase–conjugated goat anti-mouse IgG antibody. Immunoreactive protein was visualized by using Renaissance chemiluminescence reagent.

Protein kinase C kinase assay. Rh1 cells grown in MN2E were exposed to 0.1% DMSO or calphostin-C (1.2 μmol/L) for 2 h and then stimulated with IGF-I (10 ng/mL) or PMA (1 μmol/L) for 30 min. We then used the protein kinase C (PKC) assay kit (Upstate Biotechnology) according to the manufacturer's instructions to analyze the amount of activated PKC (α, βII, and γ and ε, θ, λ, μ, δ, and ζ). The amount of incorporated radioactivity into the substrate was determined by scintillation counting. To calculate the actual PKC activity, background radioactivity associated with control IgG antibody immunoprecipitated samples was subtracted. The kinase assay was repeated three times.

Plasmids and transfection. The control vector pEGFP-N1 and the test plasmids, pEGFP-Bad (wild-type), or the same construct with a single mutation (Ser^{112}Ala; Ser^{136}Ala; Ser^{155}Ala), double mutations ($Ser^{112/136}$Ala; $Ser^{112/155}$Ala; $Ser^{136/155}$Ala), and pEGFP-Bad (triple mutant; all three serine sites mutated to alanine) used for this study have been described previously (21). Cells were transfected using the FuGENE6 according to the manufacturer's instructions. Stable expression of single or double mutants of Bad was confirmed by Western blot analysis using phosphospecific antibodies (data not shown).

Small interfering RNA experiments. Cells growing in McCoy's 5A medium with 10% fetal bovine serum were transfected with small interfering RNA (siRNA) control, siBad, si-Bid, siPKC-μ, siPKC-ε, siPKC-θ, siAkt1, or siAkt2 using Lipofectamine 2000. Cells were then incubated at 37°C for 72 h, and the levels of protein expression of Bad, Bid, PKC-μ, PKC-ε, PKC-θ, Akt1, or Akt2 were analyzed by Western blot using their respective antibodies.

Binding of Bad to 14-3-3. Rh1, Rh1/GFP-Bad (wild-type), or Rh1/GFP-Bad (triple mutant) cells cultured in MN2E medium were stimulated with IGF-I or PMA for 30 min and lysed in 500 μL M-PER buffer as described previously and immunoprecipitated using Protein A/G Plus agarose beads. Immunoprecipitates of Bad were immunoblotted with the antibodies developed against anti–14-3-3 (σ, γ, β, ε, θ, η, and ζ).

Results

Effect of growth factors on the phosphorylation of Bad. Because phosphorylation is critical for Bad inactivation (1, 2, 22–26), we first investigated whether IGF-I (10 ng/mL), insulin (250 ng/mL), epidermal growth factor (EGF; 25 ng/mL), or platelet-derived growth factor (25 ng/mL) results in phosphorylation of Bad in Rh1 cells. As shown in Fig. 1A, phosphorylation of Bad reached the maximum at 30 min after Rh1 cells were stimulated with either IGF-I or EGF. Phosphorylated Bad-specific antibodies to Ser^{136} and Ser^{155} were not sensitive enough for detecting endogenous Bad phosphorylation. Consequently, we examined the effect of these factors in Rh1 cells engineered to overexpress Bad (Rh1/GFP-Bad). As shown in Fig. 1B, EGF stimulation led to a transient phosphorylation of Bad at Ser^{112} and Ser^{155}. In contrast, IGF-I stimulation resulted in prolonged phosphorylation at each site (Fig. 1C). Similar kinetics for Bad phosphorylation was observed in Rh30 cells stimulated with IGF-I (data not shown). These results show that IGF-I or EGF treatment results in different patterns of Bad phosphorylation at multiple sites.

IGF-I or EGF stimulation of Bad phosphorylation is via phosphoinositide 3′ kinase but not ERK1/2, mTOR, or protein kinase A. We (18) and others (27–31) have shown that phosphoinositide 3′ kinase (PI3K) mediates IGF-I–mediated antiapoptotic signals. To determine whether PI3K or ERK1/2 signaling was required for phosphorylation of each Bad site, Rh1/GFP-Bad cells were exposed to wortmannin (0.9 μmol/L) or the MEK1/2 inhibitor PD98059 (30 μmol/L) before stimulation with IGF-I (Fig. 2A) or EGF (Fig. 2B). Wortmannin attenuated the IGF-I– or EGF–induced phosphorylation of Bad at each site, whereas inhibition of MEK1/2 did not. PD98059 did inhibit the IGF-I– and EGF–dependent activation of $p90^{RSK}$ as determined by a block in phosphorylation of

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Thr^{573} and Ser^{380} within the protein. The PKC inhibitor calphostin-C also failed to inhibit p90^{RSK} phosphorylation in response to EGF or IGF-I treatment, indicating that PKCs may not be required for the activation of p90^{RSK} (Supplementary Fig. S1A). To further determine if ERK1/2 was important for IGF-I–dependent phosphorylation of Bad, a dominant-negative form of Ras (Ras N17) was expressed, which completely inhibited Ras activation and ERK1/2 phosphorylation induced by IGF-I or EGF (18). Ras N17 failed to suppress phosphorylation of Bad (Ser^{112}) after IGF-I stimulation (Supplementary Fig. S1B).

In addition, IGF-I–induced phosphorylation of Bad (Ser^{112}) was not inhibited by rapamycin (Supplementary Fig. S1C). Further, activation of protein kinase A with forskolin, as measured by phosphorylation of CREB (inhibited by the protein kinase A inhibitor KT5720), does not result in a phosphorylation of Bad (Supplementary Fig. S1D). This result excludes protein kinase A in the IGF-I–mediated phosphorylation of Bad.

To further elucidate the role of Akt in Bad phosphorylation, Akt1 and Akt2 were silenced using siRNA specific for Akt1 or Akt2. siRNA greatly suppressed expression of Akt1 and Akt2 (Fig. 2C). However, phosphorylation of Bad (Ser^{112}) induced by IGF-I was not abrogated (Fig. 2D). Further, infection of Rh1 or Rh30 cells with "empty" replication-defective adenovirus (lacking the E1 and E3 regions), which results in massive activation of both PI3K/Akt and mitogen-activated protein kinase pathways consistent with published data (32), failed to induce phosphorylation of Bad (Ser^{112}; Supplementary Fig. S2).

IGF-I activates PKC (μ, θ, or ε) via PI3K. To determine the possible involvement of different isoforms of PKC in IGF-I signaling, Rh1 cells were preincubated in MN2E medium with 0.1% DMSO or 1.2 μmol/L calphostin-C for 2 h before they were stimulated with IGF-I or the PKC activator PMA (1 μmol/L) for 30 min. Cell lysates were then immunoprecipitated with antibodies specific for PKC-α, PKC-βII, PKC-γ, PKC-δ, PKC-ε, PKC-θ, PKC-μ, PKC-λ, or PKC-ζ, and the resulting kinase activity of the immunocomplex was determined by measuring ^{32}P phosphate transfer from ATP to peptide substrates. IGF-I treatment resulted in a significant increase in the activity of only PKC-μ, PKC-θ, and PKC-ε (Fig. 3A). PMA was used as a positive control for PKC activation. Further, as shown in Fig. 3A, activation of the three isoforms of PKC by IGF-I or PMA was effectively blocked by calphostin-C.

Wortmannin inhibited the IGF-I–induced phosphorylation of PKC-μ at Ser^{744}/Ser^{748} but not PMA-induced phosphorylation (Fig. 3B), indicating that PI3K plays an important role in IGF-I–mediated activation of PKC in Rh1 cells. To more directly assess the role of PKC in mediating Bad phosphorylation (10, 11, 33, 34), Rh1 cells grown in MN2E medium were exposed to potent inhibitors of PKC, calphostin-C (1.2 μmol/L), or chelerythrine chloride (3.5 μmol/L) for 2 h before stimulating with IGF-I for 30 min (Fig. 3C). Both calphostin-C and chelerythrine chloride suppressed IGF-I stimulation of Bad (Ser^{112}) phosphorylation but failed to block phosphorylation of Akt. PMA-induced phosphorylation of

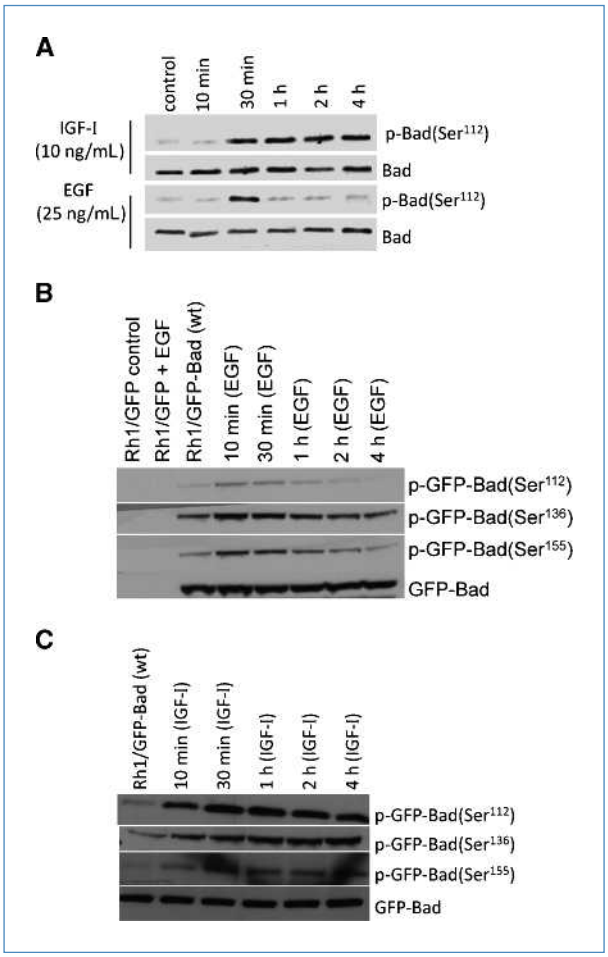

Figure 1. IGF-I induces sustained phosphorylation of Bad, while EGF induces transient phosphorylation of Bad. Cells grown in MN2E medium were stimulated with IGF-I (10 ng/mL) or EGF (25 ng/mL) for different times. Phosphorylated Bad (Ser^{112}, Ser^{136}, or Ser^{155}) was detected using phosphospecific antibodies. Identical results were obtained in at least three independent experiments. A, Rh1 cells. B and C, Rh1/GFP-Bad (wild-type) cells.

Bad at Ser^{112} in Rh1 cells (10) is also blocked by treatment with chelerythrine chloride (Fig. 3C). To test whether phosphorylation of Bad at other sites was PKC dependent, Rh1/GFP-Bad cells grown in MN2E medium were exposed to calphostin-C for 2 h before stimulating with IGF-I for 30 min. Immunoblotting revealed that calphostin-C blocked IGF-I–induced Bad phosphorylation at each site (Fig. 3D).

The simultaneous knockdown of genes for these three PKC isoforms using siRNAs resulted in severe toxicity to cells. However, individual knockdown of PKC-μ, PKC-ε, or PKC-θ by means of siRNAs failed to inhibit the phosphorylation of Bad at Ser^{112}, suggesting that the remaining PKC isoforms may be compensating for knockdown of an individual isoform (Supplementary Fig. S3).

Direct interaction between PKC-μ or PKC-ε and Bad in Rh1 cells. To test whether PKC can directly interact with Bad, Rh1 cells were stimulated with IGF-I and cell lysates

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were immunoprecipitated with anti-PKC-μ, PKC-ε, or anti-Bad antibodies and immunoblotted. The results show coimmunoprecipitation of PKC-μ or PKC-ε with Bad in Rh1 cells and provide evidence that there is a direct physical association (Fig. 4A).

Active PKC (μ, θ, or ε) can directly phosphorylate endogenous Bad at Ser^{112}. To determine whether PKC can directly phosphorylate endogenous Bad, the lysates from serum-starved Rh1 cells were immunoprecipitated with anti-Bad antibody and incubated with purified recombinant active PKC-μ, PKC-θ, or PKC-ε enzyme in a kinase buffer containing ATP (200 μmol/L). The samples were resolved and immunoblotted for phosphorylated Bad at Ser^{112} using phosphospecific antibody. IGF-I–stimulated phosphorylated Bad was used as a positive control. Results indicate that active PKC isoforms directly phosphorylate Bad at Ser^{112} (Fig. 4B). These findings suggest that PKC is a probable candidate for being the direct Bad kinase. Additional experiments were conducted to ascertain whether recombinant active PKC phosphorylates recombinant Bad. Recombinant Bad protein conjugated to agarose beads was incubated with purified active PKC-μ, PKC-θ, or PKC-ε for 30 min in an *in vitro* kinase assay. Phosphorylation of Bad was detected using phosphospecific antibodies to Ser^{112}, Ser^{136}, or Ser^{155}. As shown in Fig. 4C, recombinant PKC-μ can directly phosphorylate recombinant Bad protein at all the three sites, whereas the activity of PKC-θ or PKC-ε was weak against Ser^{155} and not detected against Ser^{136}.

IGF-I stimulates the binding of Bad to 14-3-3γ under apoptotic conditions. We tested the interaction of Bad with seven different 14-3-3 isoforms (35). Immunoblotting with antibodies specific for particular isoforms of 14-3-3 (α, β, γ, θ, η, ζ, or ε) showed that both γ and θ isoforms were expressed at high levels. We hypothesized that, under apoptotic conditions induced by rapamycin treatment, Bad would not be associated with 14-3-3γ but that the protective effects of IGF-I would stimulate binding. To test this hypothesis, Rh1 cells grown in MN2E medium were exposed to 0.1% DMSO or rapamycin (100 ng/mL) in the absence or presence of IGF-I continuously for 5 days. Lysates were prepared, and Bad was immunoprecipitated using anti-Bad antibody, resolved by SDS-PAGE, and immunoblotted using anti–14-3-3γ antibody. As shown in Fig. 4D, very little association of Bad with 14-3-3γ was seen in control cells or in the presence of rapamycin (apoptotic conditions). In contrast, treatment with IGF-I, in the absence or presence of rapamycin, resulted in a marked increase in Bad complexed with 14-3-3γ. These findings suggest a possible mechanism by which IGF-I protects the cells from apoptosis induced by rapamycin through inducing binding of Bad to 14-3-3γ.

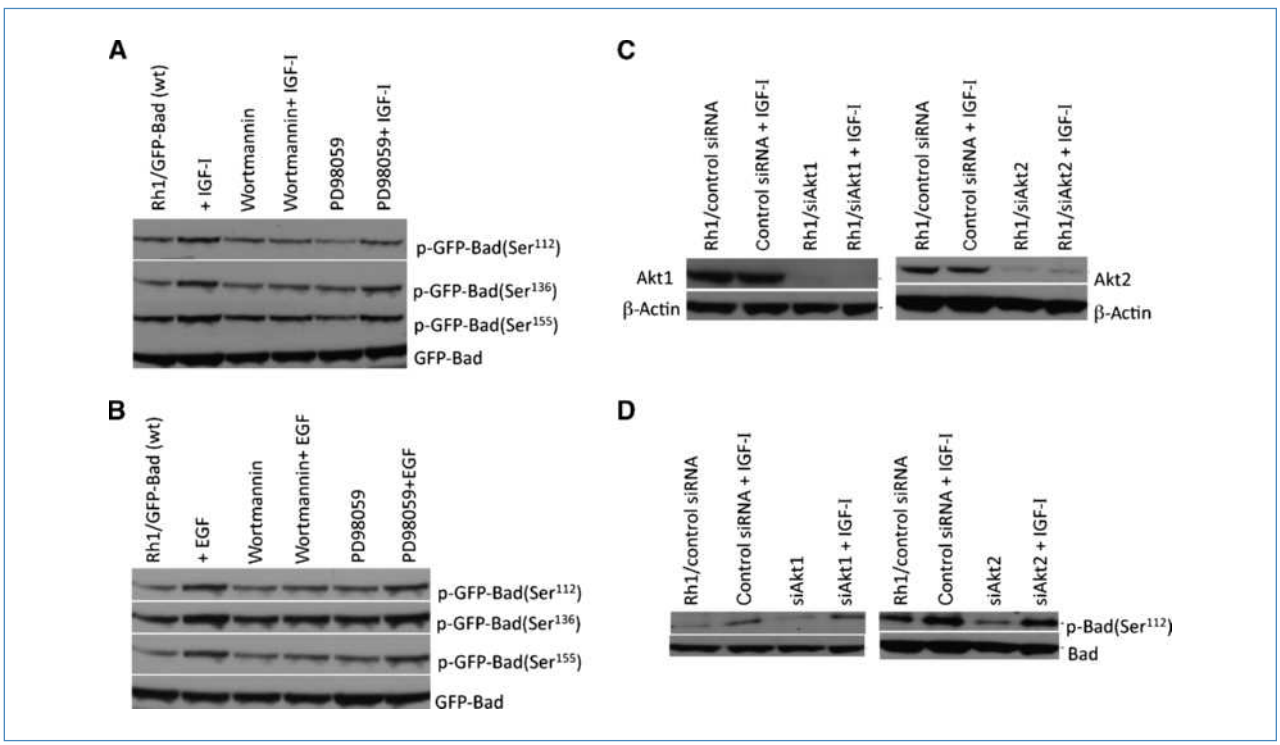

Figure 2. IGF-I phosphorylates Bad at Ser^{112} via the PI3K pathway independent of AKT. A, Rh1/GFP-Bad cells were grown in MN2E and treated with wortmannin and PD98059 for 2 h and stimulated with IGF-I for 30 min. Blots were probed with Bad phosphospecific antibodies. B, Rh1/GFP-Bad cells were treated as in A but stimulated with EGF. C, Rh1 cells were transfected with control siRNA or siRNA pools specific for Akt1 (left) or Akt2 (right). Transfected cells grown in MN2E medium were either stimulated with IGF-I or not treated. Levels of Akt1 and Akt2 were determined by immunoblotting. Actin was used as control. D, Rh1 cells were transfected and treated as in C. The levels of Bad (Ser^{112}) were detected by immunoblotting. Representative of at least three independent experiments.

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To determine whether PMA-induced phosphorylated Bad (Ser^{112}) binds to 14-3-3 proteins, Rh1 cells cultured in MN2E medium were stimulated with IGF-I or 1 μmol/L PMA for 30 min, and samples were processed as before. 14-3-3γ coimmunoprecipitated with Bad in cells stimulated with either IGF-I or PMA but not from the nonstimulated control cells (Fig. 4D).

Bad phosphorylation at any site (Ser^{112}, Ser^{136}, and Ser^{155}) facilitates IGF-I rescue from rapamycin-induced apoptosis. To address the role of Bad phosphorylation in antiapoptotic signaling, we tested the survival effect of IGF-I in rapamycin-treated Rh1 cells that were engineered to stably express pEGFP-N1 (vector control), wild-type GFP-Bad, single and double phosphorylation site mutants, and the triple mutant containing amino acid substitutions Ser^{112}Ala, Ser^{136}Ala, and Ser^{155}Ala. In preliminary experiments, we determined that Rh1/GFP-Bad (wild-type) or Rh1/GFP-Bad (triple mutant) could be stably expressed in Rh1 cells (Supplementary Fig. S4A), and calphostin-C blocked IGF-I–induced phosphorylation of Bad at each site (Supplementary Fig. S4B). As anticipated, no phosphorylation was detected in the Bad mutant where all three sites were mutated to alanine (Supplementary Fig. S4C). Overexpressed wild-type Bad bound 14-3-3γ, whereas the triple-mutant Bad failed to bind 14-3-3γ (Supplementary Fig. S4D).

The effect of rapamycin treatment on the viability of Rh1 cells expressing GFP-Bad (wild-type) or the Bad mutants was compared with the effect on the viability of an empty vector

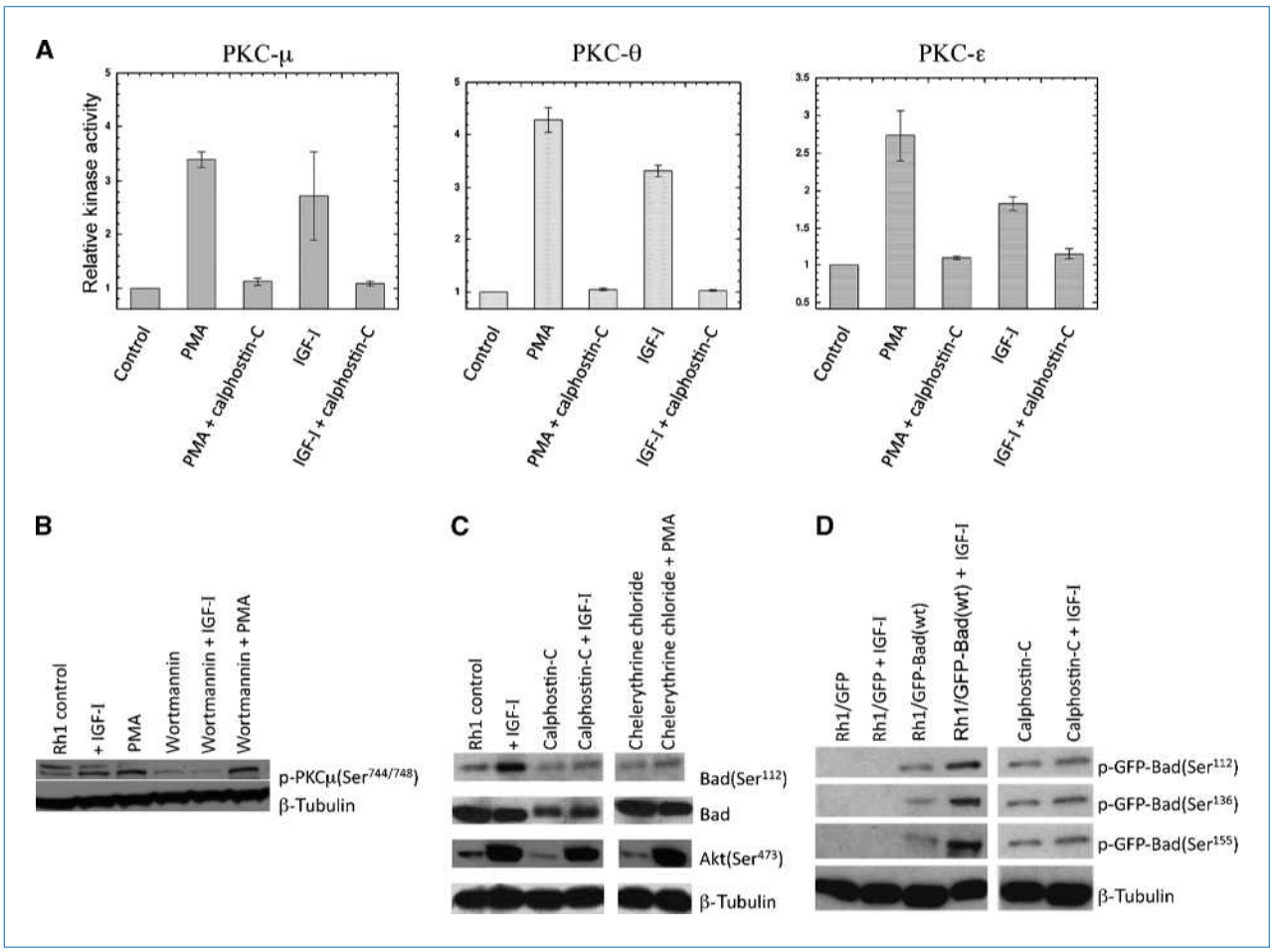

Figure 3. PKC-μ, PKC-θ, and PKC-ε are activated by IGF-I and inhibited by PKC and PI3K inhibitors. A, Rh1 cells grown in MN2E were stimulated with IGF-I or PMA for 30 min without or with calphostin-C for 2 h and immunoprecipitated using antibodies specific for PKC-μ, PKC-θ, and PKC-ε. Immunocomplexes were then used to assay PKC activity. Activation of the three isoforms of PKC by IGF-I (mean ± SD): PKC-μ (2.76 ± 0.81; $n = 3$), PKC-θ (3.31 ± 0.11; $n = 3$), and PKC-ε (1.82 ± 0.10; $n = 3$). B, Rh1 cells were grown under serum-free conditions and treated as described above. The levels of phosphorylated PKC-μ (Ser^{744}/Ser^{748}) was determined in the presence and absence of PMA and wortmannin with or without IGF-I stimulation. C, Rh1 cells grown under serum-free conditions were exposed to calphostin-C or chelerythrine chloride for 2 h and stimulated with IGF-I or PMA for 30 min. After blotting phosphospecific antibodies directed against Bad Ser^{112} and Akt Ser^{473} were used to probe the blot. Total Bad and β-tubulin were used as controls. D, Rh1/GFP or Rh1/GFP-Bad (wild-type) cells grown in MN2E medium were incubated without or with calphostin-C for 2 h and then stimulated with IGF-I for 30 min. Cell lysates were prepared followed by SDS-PAGE and Western blot analysis. Phosphospecific antibodies detected the phosphorylated signals on GFP-Bad at Ser^{112}, Ser^{136}, and Ser^{155} in IGF-I–treated Rh1/GFP-Bad (wild-type) cells. The blot was stripped and reprobed with anti–β-tubulin antibody. Representative of at least three independent experiments.

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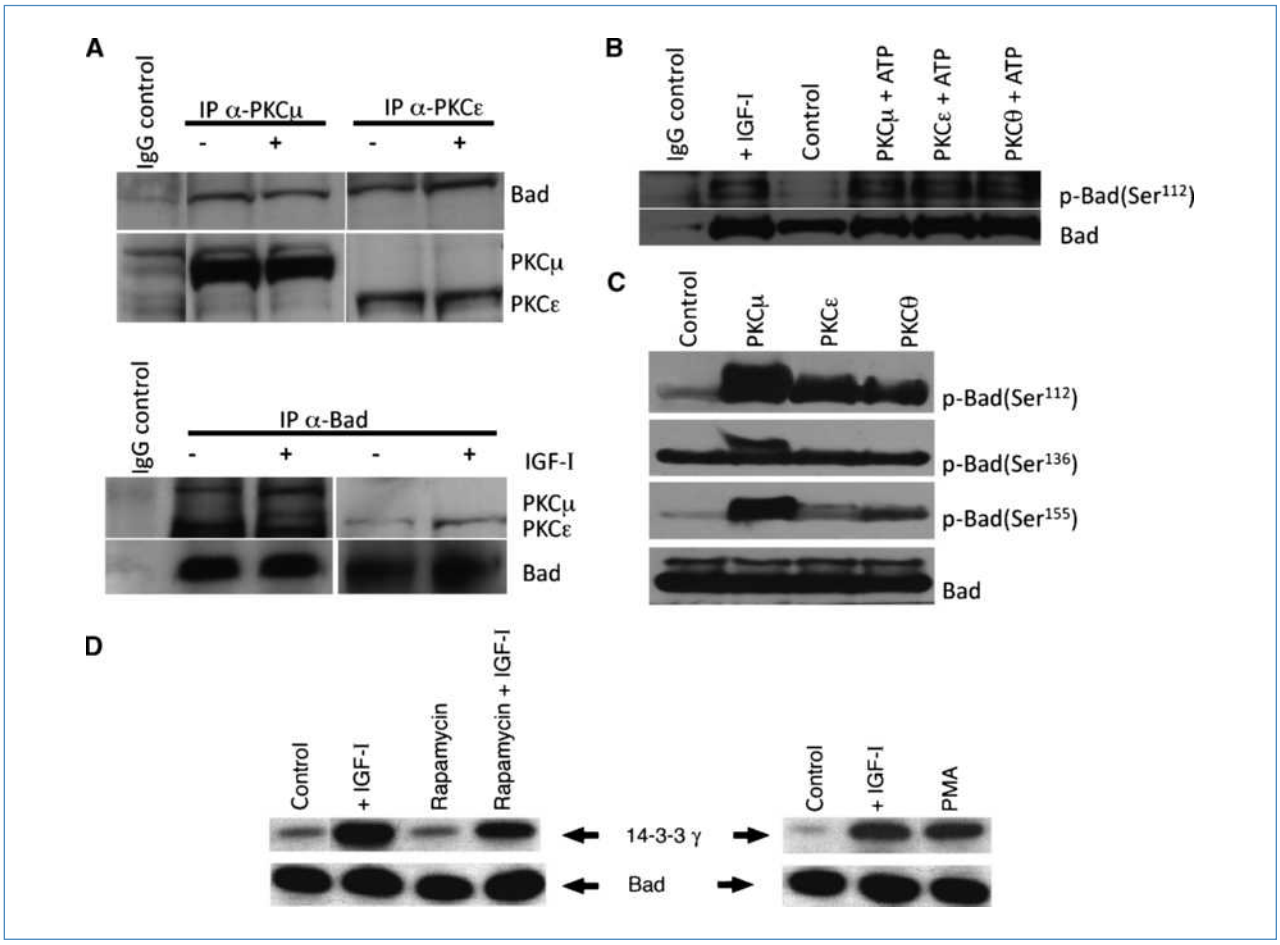

Figure 4. PKC-μ and PKC-ε immunoprecipitate with Bad and phosphorylate endogenous or recombinant Bad inducing association with 14-3-3γ. A, lysates from Rh1 cells were prepared after stimulation with IGF-I (30 min) or after no stimulation and immunoprecipitated with antibodies specific for PKC-μ, PKC-ε, or Bad. Immunoblots were probed with the anti-PKC or anti-Bad antibodies. B, endogenous Bad was immunoprecipitated from Rh1 cell lysates that had been stimulated with IGF-I or not stimulated. Lysates were incubated with recombinant PKC isozymes without (control) or with addition of ATP. Proteins were separated by SDS-PAGE electrophoresis, and immunoblots were probed with antibodies against phosphorylated Bad (Ser^{112}) or total Bad. IgG was used as a nonspecific control. C, recombinant active PKC-μ, PKC-ε, and PKC-θ phosphorylate recombinant Bad. Recombinant Bad was incubated with active recombinant PKC isozymes in the absence (control) or presence of ATP. Proteins were separated by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes. The immunoblots were probed with antibodies against phosphorylated Bad (Ser^{112}, Ser^{136}, and Ser^{155}) or total Bad. The control reaction lacked ATP. The results were similar in at least three independent experiments. D, IGF-I stimulates the binding of Bad to 14-3-3γ under conditions where rapamycin induces apoptosis. Rh1 cells were grown under serum-free conditions ± rapamycin and ± IGF-I for 5 days (left) or stimulated with PMA or IGF-I for 30 min (right). Cell lysates were immunoprecipitated using anti-Bad antibody. The precipitated proteins were resolved by SDS-PAGE and immunoblotted using anti–14-3-3γ antibody. Representative of three independent experiments.

GFP-expressing control cell line using the ApoAlert flow cytometric assay and presented in Table 1 (cells expressing single, double, or triple mutants of Bad). Expression of Bad wild-type and mutants was approximately similar (Supplementary Fig. S5). Consistent with our earlier findings, the apoptotic profile of Rh1 cells expressing pEGFP-N1 vector was almost identical with those of parental Rh1 cells, with ~25% of the empty vector–expressing cells positive for both Annexin V and propidium iodide staining. IGF-I reduced this level to ~15% to 20%. Rapamycin treatment resulted in ~60% of control cells undergoing apoptosis, and addition of IGF-I resulted in almost complete protection from apoptosis. Similar results were obtained in cells expressing single or double Bad mutants, whereas IGF-I completely protected against rapamycin-induced apoptosis. Expression of GFP-Bad (triple mutant) in Rh1 cells decreased the overall viability to a significant extent resulting in ~88% apoptosis under rapamycin-free culture conditions, and IGF-I could not reverse this effect completely (apoptotic population of 50%). This rescue probably reflects IGF-I–mediated abrogation of endogenous Bad function (~40% of cells rescued). Rapamycin treatment resulted in ~95% of cells being scored as apoptotic, and addition of IGF-I resulted in the reduction of this population to ~76%, thus exhibiting only a marginal rescue effect. Taken together, our experiments with ectopic overexpression of phosphorylation-deficient Bad mutants indicate that Bad phosphorylation at one of three serine residues is essential for the antiapoptotic effects of IGF-I.

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Knockdown of Bad results in a partial increase of cytoprotection from rapamycin-induced apoptosis. Our results indicate that IGF-I can abrogate the proapoptotic activity of Bad by inducing its phosphorylation at multiple sites in sarcoma cells. To test this, RNA interference was employed. Results show that siRNA can potently and specifically reduce Bad expression by >95%, whereas the control siRNA had no effect (Fig. 5A). To determine whether Bad is responsible for rapamycin-induced apoptosis, we exposed Rh1/sicontrol RNA or Rh1/siBad cells to rapamycin for 4 days in the presence or absence of IGF-I and determined the extent of apoptosis within each treatment population. Analysis of the results (Table 1; Fig. 5B) revealed that, in Rh1/sicontrol cells, rapamycin increased apoptosis by 31%, whereas, in Bad-deficient cells, rapamycin increased apoptosis by only 16%. In both conditions, coincubation with IGF-I almost completely protected the cells. Thus, ~50% of the apoptosis induced by rapamycin appears to be mediated through Bad.

Discussion

The specific objectives of the present study were to determine whether Bad phosphorylation is required for IGF-I rescue of Rh1 cells from rapamycin-induced apoptosis and to

Table 1. IGF-I rescue from rapamycin-induced apoptosis in Rh1 cells expressing Bad single, double, or triple phosphorylation mutants

Cell line + treatment*	Viable cells (%), mean ± SD	Apoptotic cells (%), Mean ± SD
Rh1/GFP	83.81 ± 6.53	15.91 ± 6.70
Rh1/GFP + IGF-I	89.6 ± 5.54	10.07 ± 5.93
Rh1/GFP + rapamycin	51.53 ± 11.98	48.47 ± 12.42
Rh1/GFP + rapamycin + IGF-I	86.89 ± 5.88	12.77 ± 5.99
Rh1/GFP-Bad (wild-type)	74.50 ± 2.29	25.50 ± 2.52
Rh1/GFP-Bad (wild-type) + IGF-I	83.50 ± 4.27	16.50 ± 0.50
Rh1/GFP-Bad (wild-type) + rapamycin	40.00 ± 8.32	60.00 ± 9.40
Rh1/GFP-Bad (wild-type) + rapamycin + IGF-I	79.50 ± 9.80	20.50 ±1.32
Rh1/GFP-Bad $Ser^{112}Ala$	68.50 ± 9.90	31.50 ± 2.52
Rh1/GFP Bad $Ser^{112}Ala$ + IGF-I	84.00 ± 7.00	16.00 ± 2.55
Rh1/GFP-Bad $Ser^{112}Ala$ + rapamycin	37.00 ± 2.65	63.00 ± 2.20
Rh1/GFP-Bad $Ser^{112}Ala$ + rapamycin + IGF-I	84.50 ±2.29	15.50 ± 1.50
Rh1/GFP-Bad $Ser^{136}Ala$	64.00 ± 8.72	36.00 ± 3.72
Rh1/GFP-Bad $Ser^{136}Ala$ + IGF-I	85.00 ± 4.36	15.00 ± 2.61
Rh1/GFP-Bad $Ser^{136}Ala$ + rapamycin	38.00 ± 3.08	62.00 ± 1.74
Rh1/GFP-Bad $Ser^{136}Ala$ + rapamycin + IGF-I	80.00 ± 6.21	20.00 ± 5.00
Rh1/GFP-Bad $Ser^{155}Ala$	66.00 ± 5.29	34.00 ± 6.00
Rh1/GFP-Bad $Ser^{155}Ala$ + IGF-I	80.00 ± 8.00	20.00 ± 5.00
Rh1/GFP-Bad $Ser^{155}Ala$ + rapamycin	38.00 ± 7.21	62.00 ± 4.80
Rh1/GFP-Bad $Ser^{155}Ala$ + rapamycin + IGF-I	80.00 ± 7.00	20.00 ± 4.00
Rh1/GFP-Bad $Ser^{112/136}Ala$	67.50 ± 1.50	32.50 ± 1.50
Rh1/GFP-Bad $Ser^{112/136}Ala$ + IGF-I	85.00 ± 7.00	15.00 ± 2.00
Rh1/GFP-Bad $Ser^{112/136}Ala$ + rapamycin	35.00 ± 3.00	65.00 ± 9.17
Rh1/GFP-Bad $Ser^{112/136}Ala$ + rapamycin + IGF-I	82.50 ± 3.50	17.50 ± 3.50
Rh1/GFP-Bad $Ser^{112/155}Ala$	57.00 ± 3.00	43.00 ± 2.87
Rh1/GFP-Bad $Ser^{112/155}Ala$ + IGF-I	84.50 ± 2.93	15.50 ± 1.77
Rh1/GFP-Bad $Ser^{112/155}Ala$ + rapamycin	34.50 ± 1.50	65.50 ± 2.50
Rh1/GFP-Bad $Ser^{112/155}Ala$ + rapamycin + IGF-I	80.50 ± 2.50	19.50 ± 2.50
Rh1/GFP-Bad $Ser^{136/155}Ala$	53.50 ± 2.35	46.50 ± 2.35
Rh1/GFP-Bad $Ser^{136/155}Ala$ + IGF-I	85.00 ± 2.65	15.00 ± 2.65
Rh1/GFP-Bad $Ser^{136/155}Ala$ + rapamycin	35.00 ± 3.00	65.00 ± 7.00
Rh1/GFP-Bad $Ser^{136/155}Ala$ + rapamycin + IGF-I	80.00 ± 8.50	20.00 ± 2.56
Rh1/GFP-Bad (triple mutant)	12.00 ±4.00	88.00 ± 6.50
Rh1/GFP-Bad (triple mutant) + IGF-I	50.00 ± 2.70	50.00 ± 3.20
Rh1/GFP-Bad (triple mutant) + rapamycin	5.00 ± 1.80	95.00 ± 5.60
Rh1/GFP-Bad (triple mutant) + rapamycin + IGF-I	24.00 ± 3.70	76.00 ± 6.00

*IGF-I, 10 ng/mL; rapamycin, 100 ng/mL, Annexin V–negative, propidium iodide–positive, <1.5%.

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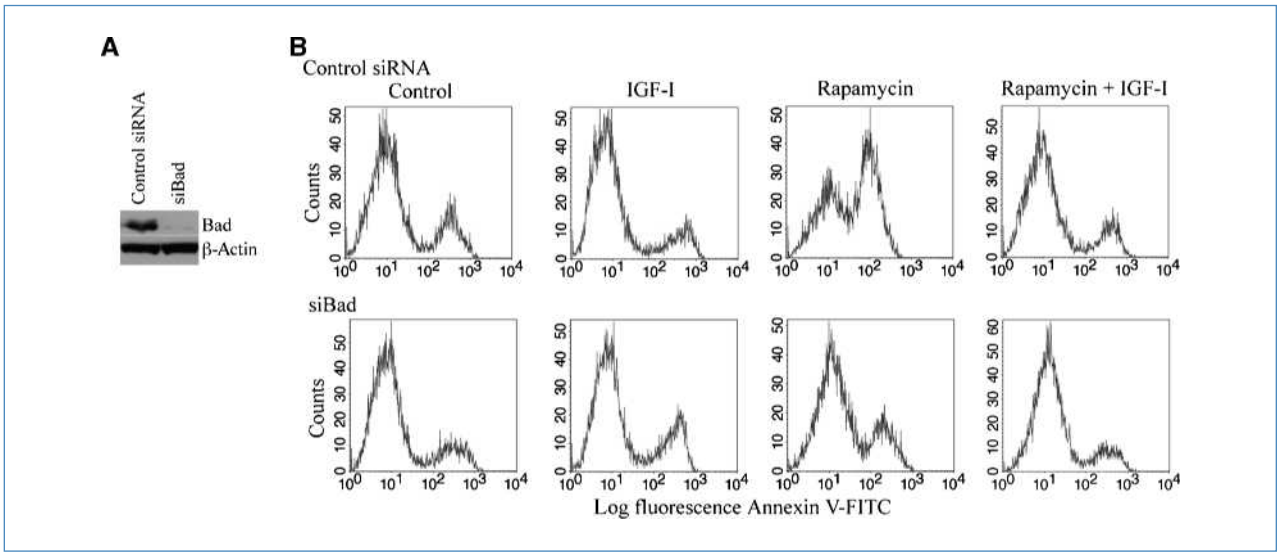

Figure 5. Downregulation of Bad protects cells from rapamycin-induced apoptosis. A, Rh1 cells were transfected with siRNA pools against Bad or control RNAi. Cells were grown for an additional 2 days, and levels of Bad were determined by immunoblotting. B, Rh1 cells transfected with control or Bad siRNA were grown in MN2E medium without treatment (control) or with IGF-I, rapamycin, or both reagents. Apoptosis was determined using the ApoAlert assay after 4 days of treatment. Similar results were obtained in three separate experiments.

define the IGF-I–Bad signaling pathway. We have dissected the signaling events leading to Bad phosphorylation at individual sites (Ser^{112}, Ser^{136}, and Ser^{155}), and our results indicate that, in response to IGF-I or EGF, endogenous Bad is phosphorylated at Ser^{112} but not significantly at Ser^{136} or Ser^{155} in Rh1 and Rh30 cells as evidenced by the failure to detect the phosphorylation sites of endogenous Bad at Ser^{136} and Ser^{155} by available phosphospecific antibodies, consistent with a previous report (36). In Rh1 cells, EGF induces only transient phosphorylation of Bad (Ser^{112}) and does not afford protection from rapamycin-induced apoptosis, whereas IGF-I stimulation leads to prolonged Bad (Ser^{112}) phosphorylation and protection from apoptosis.

The observations that PI3K inhibitors blocked the IGF-I–induced phosphorylation of Bad show that PI3K mediates IGF-I receptor-Bad signaling pathway, although phosphorylation of Akt (Ser^{473}) is not necessary for Bad phosphorylation. Endogenous Akt3 expression in Rh1 cells is not detectable by Western blot analysis. Infection of cells with replication-defective adenovirus resulted in a massive phosphorylation of Akt (Ser^{473}) without phosphorylation of Bad. These data appear to confirm that Akt signaling is not involved in the Bad phosphorylation at Ser^{112} for this cell type.

IGF-I stimulation of PKC-μ phosphorylation was inhibited by wortmannin placing PI3K upstream of PKCs in the IGF-I signaling cascade. We focused on the potential role of PKCs in IGF-I–dependent phosphorylation of Bad. PMA-induced or IGF-I–induced phosphorylation of Bad on Ser^{112} was drastically inhibited by calphostin-C or chelerythrine chloride, confirming that Bad phosphorylation at Ser^{112} is PKC dependent. *In vitro* kinase assays using recombinant active PKC (μ, θ, and ε) enzymes furnished direct evidence for the phosphorylation of both endogenous and recombinant Bad at Ser^{112}, Ser^{136}, or Ser^{155}. We found that, of the three isoforms of PKC examined, only the recombinant active PKC-μ enzyme phosphorylates all three sites of recombinant Bad, whereas PKC-θ or PKC-ε enzyme targets predominantly Ser^{112} and, to a lesser extent, Ser^{155} and showed no activity against Ser^{136} on recombinant Bad. All three isoforms of recombinant active PKC enzymes phosphorylated endogenous Bad from Rh1 cells at Ser^{112}. We also observed a direct interaction between PKC-μ or PKC-ε and Bad as evidenced by immunoprecipitation, suggesting that PKC-μ or PKC-ε may be a physiologic Bad kinase. Simultaneous silencing of PKC-μ, PKC-ε, and PKC-θ resulted in severe toxicity to Rh1 cells, but the individual knockdown of PKC-μ, PKC-ε, or PKC-θ by siRNA failed to inhibit the phosphorylation of Bad at Ser^{112}, suggesting that PKC-μ, PKC-ε, and PKC-θ isoforms may be able to compensate for one another.

Our findings that calphostin-C failed to block the IGF-I–induced phosphorylation of $p90^{RSK}$ but did block Bad phosphorylation suggest that $p90^{RSK}$ is not involved in this PKC-mediated pathway. Similarly, pharmacologic and genetic evidence suggest that ERK1/2, protein kinase A, and mTOR are also not involved in IGF-I–dependent phosphorylation of Bad.

In Rh1 cells, only 14-3-3γ was found to bind phosphorylated Bad following IGF-I stimulation. Further, our observation that IGF-I stimulates the interaction between 14-3-3γ and phosphorylated Bad under conditions where IGF-I protects against rapamycin-induced apoptosis supports a possible role for this interaction in mediating the protective effects of IGF-I. To test the hypothesis that interaction of Bad with 14-3-3γ was required for IGF-I–mediated cell rescue, we used a genetic approach. Either wild-type or phosphorylation-defective GFP-Bad (triple mutant) in which Ala residues were

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substituted for regulatory serines was expressed in Rh1 cells. Mutation of all the three serine residues in Bad to alanine abrogated its ability to be phosphorylated in response to IGF-I and completely abolished its binding with 14-3-3γ.

The results of the apoptosis assays reveal Bad as a major player in rapamycin-induced cell death and that IGF-I–mediated protection correlates well with phosphorylation and sequestration of Bad. In Rh1 cells stably expressing control vector, single or double mutants of Bad or Bad (wild-type) cells undergo apoptosis to an extent of ~25% to 50% in serum-free medium. However, addition of IGF-I resulted in essentially complete protection. These results support the contention that Bad inactivation can be effected through phosphorylation of any one of the three regulatory serines. Expression of Bad (triple mutant) offered the opportunity to determine the contribution played by Bad, as opposed to other proapoptotic molecules, in rapamycin-induced apoptosis. The viability of Bad (triple mutant) cells was reduced to a significant extent, and the apoptotic population increased to ~88% under serum-free conditions. In contrast to the phosphorylation-competent mutants of Bad, IGF-I failed to protect Bad (triple mutant) cells completely. IGF-I reduced the apoptotic population from ~88% to 50%. This modest rescue probably is due to IGF-I inducing phosphorylation and inactivation of endogenous Bad but not altering the proapoptotic activity of the triple mutant. Rapamycin treatment increased the apoptotic population from ~88% to ~95%, and IGF-I reduced this to ~76%, suggesting that it rescued the component contributed by the endogenous Bad but not that contributed by the phosphorylation-defective mutant. However, apoptosis contributed by the triple mutant makes analysis of the contribution of mutant and wild-type Bad difficult.

To better understand the effect of rapamycin alone, Bad was silenced in Rh1 cells using siRNA. Rapamycin increased apoptosis by 31% in the siRNA control cells, whereas it increased apoptosis by only 16% in siBad cells. These results suggest that Bad downregulation plays a significant role in protection from rapamycin-induced apoptosis. However, addition of IGF-I to siBad cells resulted in almost complete protection.

This may indicate that a small fraction of endogenous Bad was still functional, or an alternative mechanism, other than Bad phosphorylation, contributes to partial protection from rapamycin-induced apoptosis. The apoptotic effect of rapamycin in Rh1 cells transfected with siRNA control, siBid with or without IGF-I, was also assessed. The results reveal that Bid (37) alone failed to play any role in the apoptosis caused by rapamycin (data not shown).

In summary, we suggest that the increase in Bad phosphorylation through a PI3K/PKC-mediated pathway by IGF-I may represent an important pathway for the cytoprotective effect of this growth factor. Thus, IGF-I–induced phosphorylation of Bad by PKC and subsequent sequestration by14-3-3 proteins serves at least, in part, the mechanism by which IGF-I protects Rh1 cells against rapamycin-induced apoptosis and offers a potential mechanism by which small-molecule BH3 domain inhibitors or inhibitors of PKC may be valuable in changing the cellular response to rapamycin from cytostasis under normal physiologic conditions where IGF-I is present to apoptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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