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14-3-3 Inhibits Bad-Induced Cell Death through Interaction with Serine-136

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ABSTRACT

14-3-3 proteins are a family of multifunctional phosphoserine binding molecules that can serve as effectors of survival signaling. Understanding the molecular basis for the prosurvival effect of 14-3-3 may lead to the development of agents useful in the treatment of disorders involving dysregulated apoptosis. One target of 14-3-3 is the proapoptotic Bcl-2 family member Bad. Serine phosphorylation of Bad is associated with 14-3-3 binding and inhibition of Bad-induced cell death, but the relative contributions of the three known phosphorylation sites to 14-3-3 binding have not been established. Here we demonstrate that S136 of Bad is vital for 14-3-3 interaction, but S112 seems to be dispensable. 14-3-3/Bad interaction was strictly dependent on the presence of phosphorylated S136 in vitro, in yeast, and in mammalian cells. However, mutation of S112 did not affect 14-3-3 binding. The death caused by wild-type and S112A Bad, but not that caused by S136A Bad, could be almost completely abrogated by 14-3-3. These data support a critical role for 14-3-3 in regulating Bad proapoptotic activity. The effect of 14-3-3 on Bad is controlled largely by phosphorylation of S136, whereas S112 may represent a 14-3-3-independent pathway.

The 14-3-3 family of ubiquitously expressed, dimeric proteins consists of seven known mammalian isoforms (for review, see Fu et al., 2000). It exhibits a remarkable degree of sequence conservation, both between species and between isoforms. 14-3-3 is known for its ability to bind many different protein ligands, most of which contain phosphoserine (Muslin et al., 1996). An emerging role for 14-3-3 is as an effector of prosurvival signaling (Fu et al., 2000), suggested in part by the large number of 14-3-3 binding proteins involved in apoptosis, such as A20 (Vincenz and Dixit, 1996), ASK1 (Zhang et al., 1999), Bad (Zha et al., 1996), and FKHRL1 (Brunet et al., 1999). Experiments using dominant negative forms of 14-3-3 in cultured cells (Zhang et al., 1999) and transgenic animals (Xing et al., 2000) support this notion.

Bad is a member of the BH3-only subfamily of the Bcl-2 apoptosis-regulating proteins, which is regulated extensively by phosphorylation on serine. At least three sites on Bad can be phosphorylated in vivo, including S112, S136, and S155 of the murine protein (Zha et al., 1996; Datta et al., 2000; Lizcano et al., 2000; Tan et al., 2000; Virdee et al., 2000; Zhou et al., 2000). In the absence of phosphorylation, Bad is found localized to the mitochondria bound to Bcl-2 and Bcl-X_L, where it can induce cell death (Zha et al., 1996). When S112 and S136 of Bad become phosphorylated, Bad is found in the cytosol, bound to 14-3-3 proteins rather than Bcl-2 or Bcl-X_L (Zha et al., 1996). This form of Bad does not promote apoptosis. Mutation of any of these phosphorylation sites enhances the ability of Bad to kill cells, suggesting that phosphorylation of Bad is a critical mechanism for inhibiting its activity.

In the case of Bad, two of its three known phosphorylation sites, S112 and S136, lie within potential 14-3-3 binding sites. S155 does not possess a known 14-3-3 binding motif. Indeed, mutation of S155 has not been shown to affect the 14-3-3/Bad interaction (Datta et al., 2000), although denatured Bad phosphorylated at S155 can bind 14-3-3 (Lizcano et al., 2000). The situation for S112 and S136 is more complex. It has been reported that both sites can bind 14-3-3 and that together they may engage both binding sites on a 14-3-3 dimer (Zha et al., 1996). This has led to the frequent use of the S112,136A double mutant. However, others have reported that the S136 site may play the predominant role (Hsu et al., 1997).

Survival factor dependent kinases link Bad activity and cellular sensitivity to apoptosis to extracellular signals. Com-

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ABBREVIATIONS: PKA, protein kinase A; RSK, p90 ribosomal S6 kinase; HEK, human embryonic kidney; HMK, heart muscle kinase; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

plexity is introduced by the fact that the three phosphorylation sites of Bad are acted on largely by distinct sets of kinases. In vitro, S112 is a good substrate for protein kinase A (PKA) (Harada et al., 1999), the p21-activated kinases (Schurmann et al., 2000), and the p90 ribosomal S6 kinases (RSK) (Tan et al., 1999; Bonni et al., 1999; Shimamura et al., 2000), whereas S136 is preferentially acted on by Akt/protein kinase B (Datta et al., 1997; del Peso et al., 1997; Blume-Jensen et al., 1998) and p21-activated kinases (Schurmann et al., 2000). The recently identified S155 site can be phosphorylated by Akt, MSK1, PKA, and RSK (Datta et al., 2000; Lizcano et al., 2000; Tan et al., 2000; Virdee et al., 2000; Zhou et al., 2000). Data also exists to support the role of these kinases in phosphorylating Bad in vivo (references above and Tang et al., 2000). Each of these kinases has different regulatory mechanisms, making it likely that some survival signals will cause the phosphorylation of only one or two sites on Bad. Thus, it becomes important to examine in detail the ability of the Bad inhibitory 14-3-3 proteins to bind S112 and S136. Through the use of multiple biochemical approaches, we determined that the serine 136 epitope is both necessary and sufficient for 14-3-3 binding, whereas serine 112 plays little role. We also examined the ability of Bad mutants to cause cell death. Bad S136A was considerably more active than wild-type or S112A Bad. 14-3-3 expression prevented wild-type and S112A Bad, but not S136A Bad, from decreasing viability. We propose that inhibition of Bad by survival signaling pathways through its S136 site requires 14-3-3, but that the S112 site is 14-3-3–independent. The ability of 14-3-3 to bind a single site on Bad may allow 14-3-3 to bring additional proteins into the Bad complex.

Materials and Methods

Plasmids

For bacterial expression, bovine 14-3-3ζ (pHAF612; Fu et al., 1993) and murine Bad (Datta et al., 1997) DNA sequences were placed in pET15b and pET16b vectors (Novagen, Madison, WI), creating histidine-tagged fusions. LexA DNA binding domain fusions with Bad (H. Y., S. C. M., H. Wang, and H. F., in preparation) for use in the yeast two-hybrid system were created in pEG202 (Gyuris et al., 1993). pHAF633 (Zhang et al., 1997) is a B42 transcriptional activation domain/14-3-3ζ expressing construct derived from pJG4–5. For expression in mammalian cells, the Bad, HA-mΔ4-129Akt, and FLAG-tagged 14-3-3ζ plasmids described previously (Datta et al., 1997) were used.

Cell Culture

HEK293, HeLa, and COS-7 cells were grown in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) containing 10% fetal calf serum (Atlanta Biologicals, Atlanta, GA). Transfection was accomplished using the calcium phosphate precipitation method (Chen and Okayama, 1987) or using FuGENE 6 cationic lipid reagent (Roche Molecular Biochemicals, Indianapolis, IN).

Protein Expression and Purification

14-3-3ζ and Bad were expressed as His_6-tagged fusions in *Escherichia coli* BL21(DE3) and purified on Ni^{+2} charged iminodiacetic acid Sepharose beads as described previously (Datta et al., 1997; Zhang et al., 1997). The His_6 tag was removed from 14-3-3 by thrombin treatment.

In Vitro Bad Binding

His_6-Bad proteins were phosphorylated by heart muscle kinase (HMK; Sigma, St. Louis, MO) in 150 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4), 15 mM $MgCl_2$, and 50 μM ATP. Reactions were carried out at 30°C for 30 min. Addition of [γ-^{32}P]ATP to some reactions allowed the determination of efficiency of phosphorylation of various Bad mutants. Kinase- or mock-treated Bad (150 ng) on Ni^{2+}-charged beads was incubated with 2.5 μg of 14-3-3ζ in binding buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM $Na_4P_2O_7$, 0.2% Nonidet P-40, 1 g/l BSA, and 1 mM phenylmethylsulfonyl fluoride; final volume, 300 μl). After incubating 2 h at 4°C on a rotating platform, the beads were washed twice with binding buffer and bound proteins were analyzed by Western blot.

Yeast Two-Hybrid Assay

The yeast two-hybrid interaction trap system (Gyuris et al., 1993) was used essentially as described previously (Zhang et al., 1997).

Immunoprecipitation

Forty-eight hours after transfection, 8×10^5 HEK293 cells were resuspended in lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% Nonidet P-40, 5 mM NaF, 5 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 10 mg/l aprotinin, 10 mg/l leupeptin, and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 1 h, and centrifuged to remove insoluble materials. The resulting lysates were precleared by incubation with 50 μl of 50% protein A Sepharose 4FF (Amersham Pharmacia Biotech, Piscataway, NJ). Bad (C-20) antibody (10 μl; Santa Cruz Biotechnology, Santa Cruz, CA) was added, and 1.5 h later, 50 μl of 50% protein A Sepharose was added. This mixture was rocked gently for 1.5 h before the beads were washed three times with ice-cold lysis buffer and once with 50 mM HEPES, pH 7.5. Bound proteins were eluted by boiling and analyzed by Western blot.

Peptide Binding Assays

High-performance liquid chromatography-purified R18 (PHCVPRDLSWLDLEANMCLP), pS-Bad-112 (AMETRSRHS[pS]YPAGTEE), and pS-Bad-136 (LSPFRGRSR[pS]APPNLWA) peptides were obtained from the Emory University Microchemistry Facility. These peptides were covalently immobilized by their N termini on NHS activated Sepharose columns (Hi-Trap NHS; Amersham Pharmacia Biotech) following the manufacturer's protocol. After coupling, the columns were cut open and the peptide-conjugated resin was used. Binding reactions consisted of 15 μl of 50% peptide beads, 100 nM 14-3-3ζ, and various concentrations of free peptides in binding buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% Nonidet P-40, 0.1% BSA) at a total volume of 250 μl. Reactions were rocked gently at 4°C for 1.5 h before washing three times with binding buffer. Bound 14-3-3 was eluted by boiling, separated by SDS-PAGE, and visualized by silver staining.

Cell Death Assays

Attachment-Based Viability Assay. COS-7 cells were grown in 24-well plates and transfected with a lacZ marker and various test

plasmids, using at least three wells for each condition (Miura and Yuan, 2000). At various time points, samples were washed twice with PBS to remove floating and loosely attached cells, then lysed with 200 μl per well of Z buffer (100 mM Na_2HPO_4, pH 6.95, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, and 0.2% Nonidet P-40). After addition of 50 μl 1 g/l chlorophenol red-β-D-galactopyranoside (Roche Molecular Biochemicals, Indianapolis, IN) the solutions were transferred to a microtiter plate and $A_{550\ nm}$ was determined using a kinetic plate reader (Molecular Devices, Sunnyvale, CA). β-Galactosidase activity, corresponding to viability, was determined as the slope of the best-fit line to the observed $A_{550\ nm}$ versus time curve.

Cell Morphology-Based Assay. COS-7 or HeLa cells were seeded in 35-mm dishes and transfected as for the attachment based assay. At 24 h after transfection, cells were fixed and stained for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Stained cells were counted in a blind fashion, with dead cells distinguished by their rounded, blebbed appearance. At least 500 cells were counted for each sample.

DNA Content Assay. COS-7 cells were transfected with various test DNAs in combination with a farnesylated enhanced green fluorescent protein marker (pEGFP-F; CLONTECH, Palo Alto, CA) (Amarante-Mendes et al., 1998). Twenty-four hours later, cells were trypsinized, washed with PBS (130 mM NaCl, 20 mM $NaPO_4$, pH 7.5), and fixed in ethanol. Samples were stored at 4°C overnight to allow small fragments of DNA, produced as cells undergo apoptosis, to diffuse out of the cells. After washing with 1% BSA/PBS, the cells were treated with RNase A (1 g/l) and stained with propidium iodide (50 μg/ml) for 30 min at room temperature before assaying by flow cytometry. Data were analyzed using WinMDI v2.8 (J. Trotter; Scripps Research Institute, La Jolla, CA) to exclude debris, clumps, and nontransfected cells. Apoptotic cells are recognized by their hypodiploid DNA content.

Results

Serine 136, but Not Serine 112, of Bad Is Essential for 14-3-3 Binding. Although Bad has two phosphorylation sites that lie within potential 14-3-3 binding motifs, distinct sets of kinases are thought to be responsible for phosphorylating them. Because of this, it is possible that the S112 and S136 sites of Bad will not share the same phosphorylation state. Thus, it is vital to determine the relative importance of these phosphoserines for 14-3-3 interaction. To examine this issue, we turned to an in vitro binding assay using Bad and 14-3-3ζ purified from *E. coli*. Untreated recombinant Bad was unable to pull down recombinant 14-3-3 (Fig. 1A). This observation is probably explained by the absence of significant serine kinase activity in *E. coli*. Bad protein was then treated with heart muscle kinase (HMK), the catalytic subunit of PKA purified from bovine heart, before performing the assay. HMK treatment resulted in the phosphorylation of at least three residues on Bad, including S112, S136, and S155, as demonstrated by blotting with phosphospecific antibodies (Fig. 1B). Wild-type, phosphorylated Bad protein was capable of binding 14-3-3ζ (Fig. 1A), supporting the idea that the 14-3-3/Bad interaction is direct. Examination of the binding of S112A and S136A mutant Bad proteins yielded surprising results. Mutation of S136 to alanine caused a complete loss in the ability of Bad to pull down 14-3-3, even though the mutant protein could be phosphorylated at S112 and S155. In contrast, the S112A Bad protein bound 14-3-3 to an extent indistinguishable from that of wild-type.

The dependence on serine 136 for 14-3-3/Bad interaction is not limited to in vitro systems. Wild-type Bad can bind 14-3-3 in *Saccharomyces cerevisiae* as determined by yeast two-hybrid measurements (Fig. 2A), which is consistent with a previous report (Hsu et al., 1997). The requirement for an intact serine 136 and the lack of effect of serine 112 mutation were completely recapitulated in this assay. None of the Bad constructs used induced lacZ reporter gene expression in the absence of 14-3-3 (data not shown), indicating that the effects of Bad mutations were caused by their relative 14-3-3 binding abilities rather than through direct transcriptional activation. We also examined the 14-3-3/Bad interaction in transfected HEK293 cells. Bad S112A immunocomplexes contained the same amount of 14-3-3 seen in wild-type Bad precipitates (Fig. 2B), whereas S136A Bad was unable to associate detectably with 14-3-3. HEK293 cells do contain a serum dependent S112 kinase activity (Fig. 2C), which can be enhanced by treatment with the PKA activator dibutyryl-cAMP or by coexpression of constitutively active Akt. This suggests that the lack of effect of the S112A mutation is not caused by insufficient phosphorylation of S112. Taken together, these data indicate that the S136 site of Bad is necessary for 14-3-3 interaction, whereas the S112 site is not critical.

The Serine 136 Epitope of Bad Is Sufficient for 14-3-3 Interaction. We desired to test the Bad binding site preference of 14-3-3 using an assay that would avoid the potential complications of using mutated proteins. To accomplish this end, 17-mer peptides derived from the Bad sequences around S112 and S136 were obtained. These peptides were chemi-

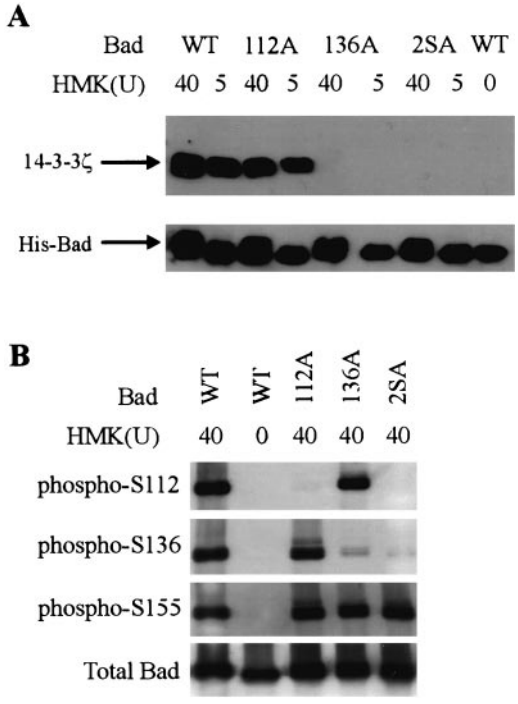

Fig. 1. Relative contributions of the S112 and S136 epitopes of Bad to 14-3-3 binding. A, in vitro binding of 14-3-3 to Bad. Recombinant His_6-Bad was phosphorylated with HMK, mixed with 14-3-3ζ, and pulled down using Ni^{2+} beads. Bead-bound Bad and coprecipitated 14-3-3 were visualized by Western blot with anti-Bad or anti-14-3-3 serum (sc-943 [Santa Cruz Biotechnology] and 1002_3, respectively). 2SA indicates the S112,136A mutant of Bad. B, phosphorylation of Bad by HMK. His_6-Bad phosphorylated with 40 U of HMK was tested for site specific phosphorylation by Western blot using phospho-S112, phospho-S136, and phospho-S155 antibodies (9291, 9295, and 9297, respectively; Cell Signaling Technology, Beverly, MA).

cally synthesized with phosphoserine in the appropriate sites (pS112 or pS136, respectively) and were conjugated to Sepharose beads at approximately equal levels. The beads were then incubated with 14-3-3ζ protein, and bound materials were analyzed by SDS-PAGE. Whereas the pS-Bad-136 peptide beads were capable of binding 14-3-3, the pS-Bad-112 peptide beads were not (Fig. 3A). Thus, under these conditions, it seems that 14-3-3 has a much higher affinity for the Bad S136 binding site than the one at S112.

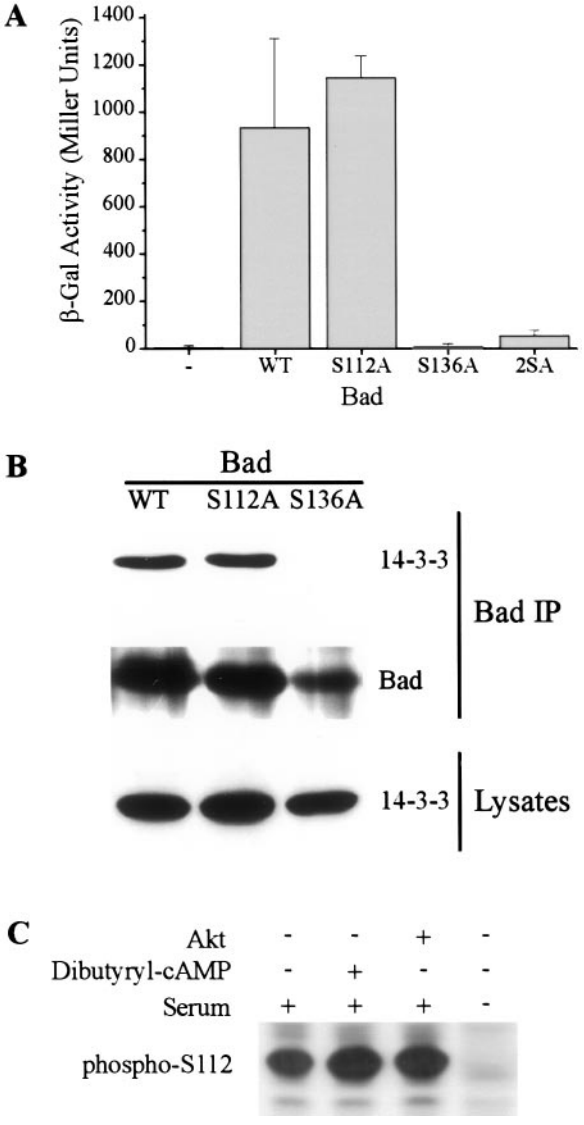

Fig. 2. Comparison of 14-3-3 binding to S112 and S136 Bad mutants in cells. A, 14-3-3 interacts with Bad in the yeast two-hybrid system. Yeast strain EGY48 containing plasmids coding for B42–14-3-3ζ and for various LexA-Bad mutants as shown were grown in synthetic liquid medium containing galactose. Cells were pelleted, lysed, and assayed for β-galactosidase activity using chlorophenol red-β-D-galactopyranoside as a substrate. Average β-galactosidase activity (± S.E.M.; $n = 3$), which is dependent on the strength of the Bad/14-3-3 interaction, is shown in Miller units. B, Bad/14-3-3 complex formation in HEK293 cells. After transfection of plasmids coding for Bad, FLAG-tagged 14-3-3ζ and constitutively active Akt cells were lysed and Bad was immunoprecipitated from the lysates using an anti-Bad antibody (sc-943). Bad and 14-3-3 expressed in the lysates or precipitated by the IP were revealed by Western blot using anti-Bad (sc-941; Santa Cruz Biotechnology) and anti-FLAG (M2; Sigma), respectively. C, phosphorylation of serine 112 of Bad. HEK293 cells were transfected with wild-type Bad and constitutively active Akt as indicated. Twenty-four hours after transfection, the medium was replaced with fresh medium containing 10% serum, medium containing serum and 100 μM dibutyryl-cAMP (Sigma), or serum-free medium. Twenty-four hours later, the cells were lysed, and lysates were separated by SDS-PAGE. A Western blot using phospho-S112 antibody (9291) is shown.

Because 14-3-3 ligands share the 14-3-3 amphipathic groove as a primary binding site (Yaffe et al., 1997; Zhang et al., 1997; Petosa et al., 1998; Wang et al., 1998), it is possible to use competition assays to measure peptide binding (Muslin et al., 1996). We used a nonphosphorylated 14-3-3 binding peptide known as R18 (Petosa et al., 1998; Wang et al., 1999) as the immobilized ligand in these experiments. R18 was coupled to Sepharose in an identical fashion as the Bad-derived peptides, and these beads were shown to bind 14-3-3ζ (Fig. 3A). R18 beads were then incubated with 14-3-3 and various concentrations of free pS-Bad-112 or pS-Bad-136 peptides. R18 bound robustly to 14-3-3 even in the presence of 50 μM pS-Bad-112 peptide (Fig. 3B). In contrast, concentrations of pS-Bad-136 peptide as low as 1 μM were able to almost completely abolish the 14-3-3/R18 interaction. It seems then that the serine 136 epitope of Bad is not only necessary for 14-3-3 binding but also sufficient to drive 14-3-3/Bad interaction. The serine 112 site seems to play at most an accessory role in modulating 14-3-3 binding.

Mutation of Serine 136 Enhances Bad Induced Cell Death. Having determined that there is a selective 14-3-3 binding defect in Bad S136A relative to S112A, we then examined the cell death caused by these mutants to determine whether this difference had functional consequences. Expression of wild-type Bad caused a dose- (Figs. 4, A and B) and time-dependent (Fig. 4C) decrease in the viability of COS-7 cells. Similar results were seen in HeLa cells as well (data not shown). Mutation of the serine 112 phosphorylation site had relatively small effects on the ability of Bad to cause death (Figs. 4, A and B) but had essentially no effect on the kinetics of death (Fig. 4C). In contrast, Bad S136A possessed increased potency and efficacy compared with wild-type or S112A Bad and also seemed to kill cells more quickly, although this may be a reflection of its ability to reduce viability more than wild-type Bad at later time points. This occurred despite the lower expression of Bad S136A relative to wild-type or S112A in COS-7 cells (Fig. 4E). These data support the concept that in COS-7 cells, the 14-3-3 binding

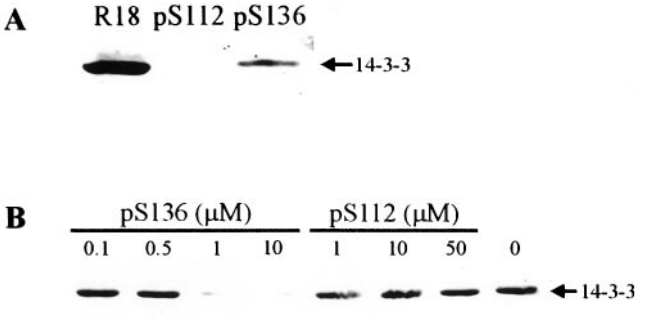

Fig. 3. Interaction of 14-3-3 with Bad derived phosphopeptides. A, direct binding of 14-3-3 to peptide-coupled beads. Synthetic phosphopeptides derived from the S112 or S136 sites of Bad or a positive control peptide (R18) were covalently bound to Sepharose beads. 100 nM 14-3-3ζ dimer was incubated with the beads and the bound 14-3-3 was visualized by SDS-PAGE and silver staining. B, competition of Bad-derived peptides for 14-3-3 interaction. 14-3-3ζ was incubated with R18 conjugated Sepharose beads as in A, but in the presence of varying amounts of free pS-Bad-112 or pS-Bad-136 peptides.

site of Bad is a key component of a prosurvival regulatory pathway, whereas the S112 pathway modulates Bad activity in a 14-3-3-independent manner.

14-3-3-Mediated Inhibition of Bad-Induced Cell Death Requires an Intact Serine 136. Although the pronounced ability of S136A Bad to kill cells supports a critical role for 14-3-3 in preventing Bad-induced cell death, we wished to examine this in a more direct fashion. Coexpression of 14-3-3 with Bad in COS-7 cells had essentially no effect on cell viability (Fig. 5A). We reasoned that this could be due to a lack of kinase activity, so constitutively active Akt was added. In the absence of transfected 14-3-3, Akt was able to partially prevent Bad-induced cell death (Fig. 5A). Increasing the amount of Akt did not change the level of rescue (data not shown), which supports the idea that Akt phosphorylation alone is not sufficient to block the proapoptotic activity of Bad. It seemed likely that Akt allowed Bad to recruit endogenous 14-3-3. Consistent with this hypothesis, in the presence of Akt, a dose-dependent increase in viability was seen upon 14-3-3 transfection (Fig. 5A). The combination of Akt and 14-3-3 was able to completely restore viability to near the level of cells not transfected with Bad. As shown in Fig. 5B, Bad S136A was refractory to the effects of Akt and 14-3-3, but Bad S112A behaved identically to wild-type. These results suggest that 14-3-3 is directly involved in the inhibition of Bad-induced cell death through its ability to bind to phosphorylated serine 136.

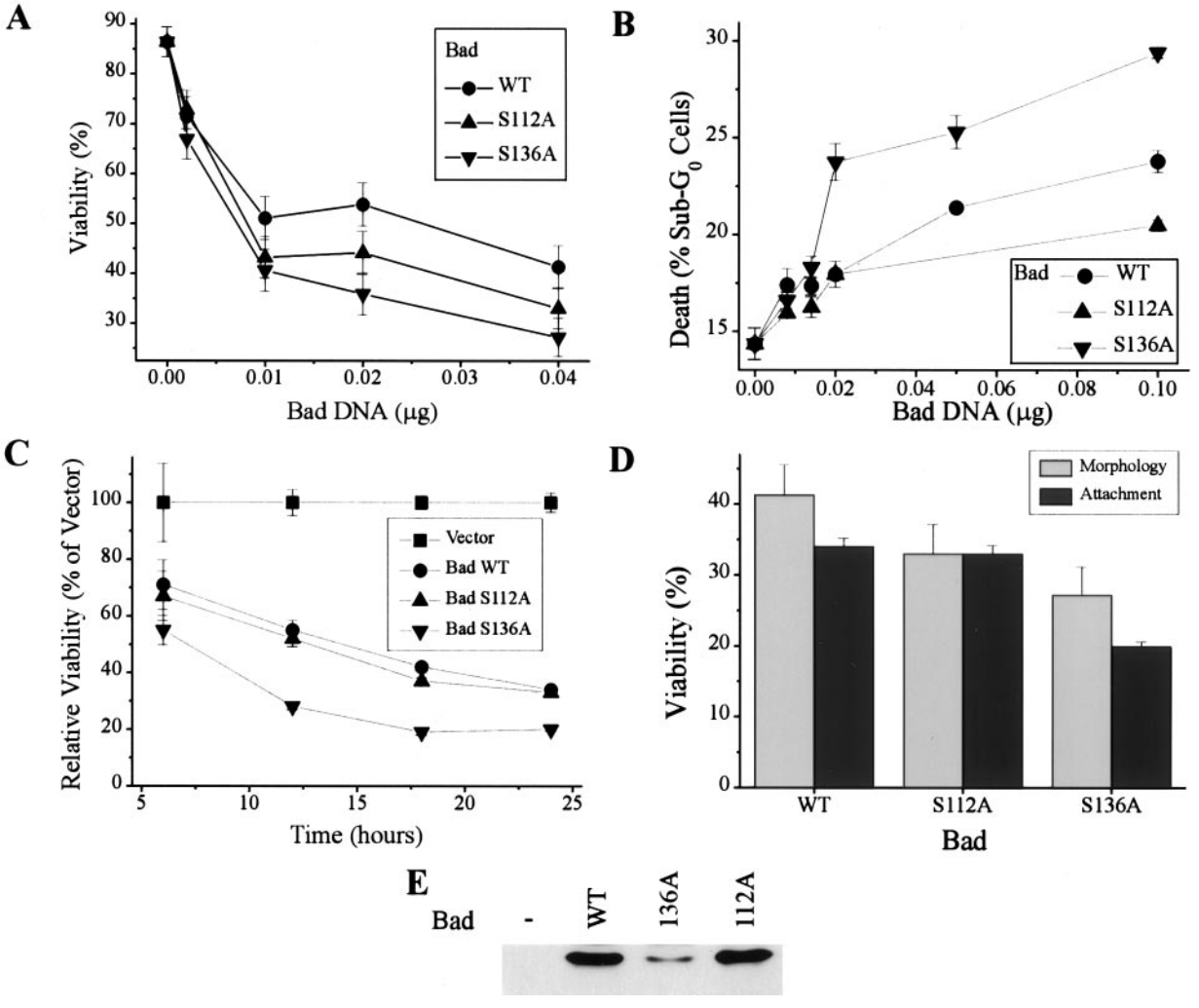

Fig. 4. Effect of Bad S112A and S136A on cell death. A, dose-dependence of Bad-induced killing. COS-7 cells were transfected with increasing amounts of plasmids coding for Bad along with a lacZ marker. Twenty-four hours later, cells were fixed and stained for β-galactosidase. Transfected cells were scored as live or dead based on their morphology. At least 500 cells were counted for each sample. Error bars represent 95% confidence intervals. B, dose dependence of Bad-induced DNA fragmentation. COS-7 cells were transfected as in A, except that EGFP-F was used as a marker. Twenty-four hours later, cells were fixed in ethanol, DNA was stained with propidium iodide, and DNA content was measured on a flow cytometer. The fraction of transfected cells with hypodiploid DNA content ($SubG_0$), which are apoptotic, is shown as mean ± S.E.M. ($n = 2$). C, time dependence of Bad-induced cell death. COS-7 cells were transfected with a lacZ marker and 0.04 μg of Bad DNA. At the indicated times, dead cells were gently washed away and the live, attached cells were assayed for β-galactosidase. Data are shown relative to vector samples to control for increasing β-galactosidase expression over the course of the experiment, and represent mean ± S.E.M. ($n = 3$). Loss of viability results in decreases in the relative β-galactosidase activity caused by cessation of protein synthesis and detachment from the culture dish. D, comparison of results from the morphology- and attachment-based viability assays. COS-7 cells transfected with 0.04 μg of Bad DNA were harvested 24 h after transfection using the two different methods described in A and C above. The results of these assays correlate well. E, expression of Bad mutants. Lysates from COS-7 cells transfected with 2 μg of hemagglutinin epitope tagged wild-type, S112A, or S136A Bad DNA were separated by SDS-PAGE. Bad expression was detected by immunoblot with an anti-HA antibody (12CA5). This blot represents unusually good expression of the S136A Bad mutant.

Discussion

Our results support the hypothesis that the interaction of Bad with death inhibitory 14-3-3 proteins is mediated primarily by S136, one of Bad's three known serine phosphorylation sites. As with most other 14-3-3 ligands, phosphorylation of Bad is necessary for 14-3-3 binding. Despite the fact that S112, S136, and S155 of Bad could be phosphorylated in vitro, we were unable to detect any effect of mutating S112 on 14-3-3ζ binding. On the other hand, S136A Bad was unable to bind 14-3-3 in vitro, in yeast, or in mammalian cells. Experiments with synthetic peptides demonstrated that the sequence surrounding S136 was sufficient for 14-3-3 binding, whereas the analogous S112-derived peptide was inactive. It has been reported that phosphorylation of denatured Bad at S155 can induce 14-3-3 binding (Lizcano et al., 2000). In contrast, mutation of S155 does not affect 14-3-3 binding (Datta et al., 2000), and we were unable to demonstrate 14-3-3 binding in vitro to S136A Bad phosphorylated on S155 (Fig. 1). Together, these data suggest that although phosphorylation of S112 and S155 could possibly have a modulatory role in binding 14-3-3, the S136 site of Bad is both necessary and sufficient to drive the 14-3-3/Bad interaction.

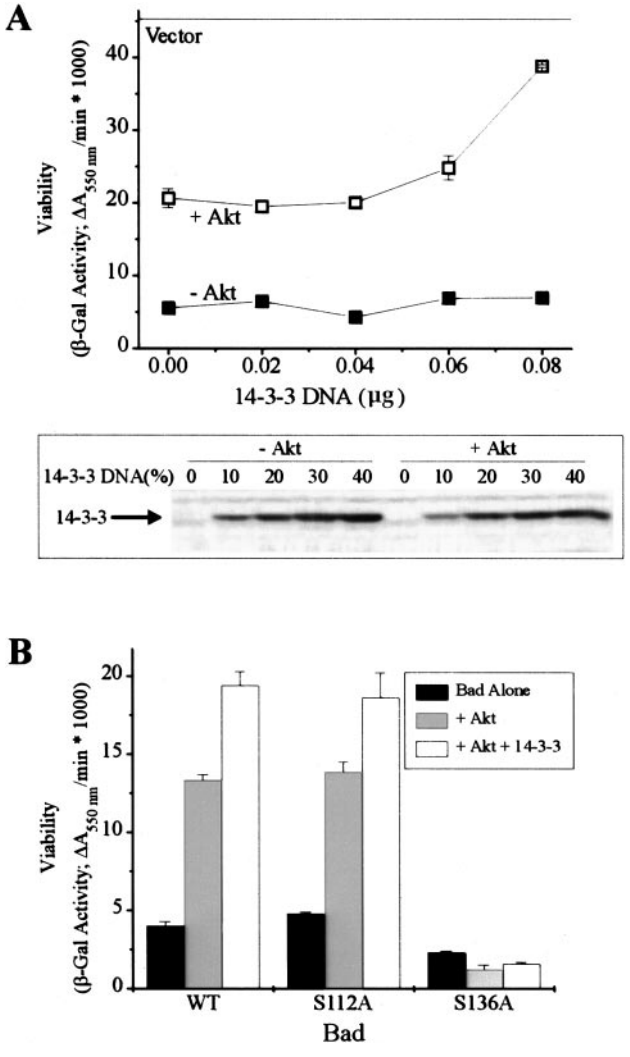

Fig. 5. Effect of 14-3-3 on Bad-induced cell death. A, blockade of Bad proapoptotic activity by 14-3-3 and Akt. COS-7 cells were transfected with a lacZ marker and 0.04 μg of Bad DNA, in addition to plasmids coding for FLAG-14-3-3ζ and constitutively active Akt (0.01 μg). After 45 h, β-galactosidase activity in the attached cells was measured. Results shown are mean β-galactosidase activities ± S.E.M. (n = 3). The inset shows the expression of FLAG-14-3-3ζ as determined by Western blot of lysates from parallel samples. B, effect of Akt and 14-3-3 expression on cell death induced by S112A and S136A Bad. Experiments were carried out as in A, using 0.11 μg of FLAG-14-3-3ζ DNA.

This model has profound implications for the regulation of Bad-induced apoptosis. It is widely believed that phosphorylation of Bad on any of S112, S136, or S155 can block the proapoptotic activity of Bad, although not necessarily directly. It has been proposed that both the S112 and S136 sites can mediate 14-3-3 binding, and that they are both required for full inhibition of Bad by 14-3-3 (Zha et al., 1996). Indeed, mutation of either S112 or S136 to alanine can amplify the proapoptotic activity of Bad. However, we show that in COS-7 cells S136A is a more potent and efficacious inducer of cell death than S112A, which reflects the relative importance of these sites in 14-3-3 binding. When the effect of 14-3-3 was tested directly, we found that S112A Bad behaved identically to wild-type, whereas S136A Bad was completely nonresponsive to 14-3-3. Thus, it seems reasonable to postulate that S136 and S112 of Bad represent the acceptors of 14-3-3-dependent and -independent survival pathways, respectively. Nonphosphorylated Bad is found localized to the mitochondria, bound to Bcl-X_L, where it can exert its proapoptotic effect. Survival signals lead to the activation of S112 and/or S136 kinases, promoting the phosphorylation of Bad. In the case of S136, 14-3-3 can then be recruited to the Bad complex, which directly or indirectly causes the eventual localization of Bad to the cytosol, away from Bcl-X_L, where it is thought to be inactive. A similar process may occur downstream of S112 but independent of the presence of 14-3-3. Either pathway alone may be capable of inactivating Bad; however, they could work in tandem as a fail-safe mechanism to prevent unintentional cell death, or possibly as a means to overcome weak proapoptotic signals. The role of S155 is not shown in this model because no relevant data was presented in this report; however, recently published results place S155 downstream of S136 (Datta et al., 2000). It was proposed that phosphorylation of S136 induces 14-3-3 interaction with Bad when bound to Bcl-X_L, promoting a conformation of Bad that allows S155 phosphorylation. S155 phosphorylated Bad is inactive. Other reports have suggested that S155 phosphorylation is independent of S136 and 14-3-3 (Tan et al., 2000; Zhou et al., 2000). These models could be reconciled by differing requirements for S155 phosphorylation when Bad is free versus when Bad is bound to Bcl-X_L.

Because 14-3-3 proteins exist as dimers, the finding that they interact with only a single site on Bad raises important new issues. It has been suggested that 14-3-3 acts to protect Bad from S112 and S136 phosphatases (Zha et al., 1996). The mapping of S136 as the primary 14-3-3 binding site does not rule out this mode of action, but it is no longer obvious that S112 would be protected. In any case, the finding that phosphorylation of Bad on S136 is not sufficient to block Bcl-X_L binding in vitro implies that phosphatase protection is not the only role for 14-3-3 (Datta et al., 2000; Lizcano et al., 2000). More attractive possibilities arise from the hypothesis that 14-3-3 can act as a scaffolding protein, recruiting proteins that don't normally interact with Bad into the complex. For example, transient 14-3-3 binding to proteins anchored to the cytoskeleton or to specific subcellular compartments

may aid in the redistribution of Bad from the mitochondria upon survival factor stimulation. Additionally, because 14-3-3 is known to interact with many different kinases, it may be that phosphorylation of S136, through 14-3-3, leads to phosphorylation of other residues of Bad, as has been proposed for S155 (Datta et al., 2000). It seems most likely that 14-3-3 inhibits Bad through the use of multiple mechanisms.

Our results also raise issues regarding the role of S112 in inhibiting Bad-induced cell death and the biochemical execution of this phenomenon. We found no evidence that S112 is involved in 14-3-3 binding, the S112A mutation was less potent than S136A Bad in induction of cell death, and S112A was completely inhibited by 14-3-3. We have not directly tested the phosphorylation status of S112 in the Bad S136A mutant because of its low level of expression in COS-7 cells. However, based on data from wild-type Bad in HEK293 cells (Fig. 2C) we believe that S112 of Bad is phosphorylated under the conditions used in our cell death assays. Thus, the S112 regulatory axis for Bad is dispensable, at least under some conditions. However, others have shown that S136A Bad-induced HEK293 cell death could be prevented by expression of RSK, whereas S112A Bad could not be inhibited (Tan et al., 1999). One possible explanation for these results is that phosphorylation of S112 has no direct effect on the ability of Bad to cause apoptosis; instead, it is a prerequisite for recruitment of an inhibitory factor. Such a factor may be in short supply in some cells, essentially preventing regulation of Bad through S112. This scheme is analogous to the S136/14-3-3 regulatory axis of Bad; however, an unknown additional protein would be involved in place of 14-3-3. Clearly, the S112 pathway for control of Bad will require further study.

In summary, we have shown that the S136 epitope of Bad represents its primary 14-3-3 binding site. This is reflected in the S136A mutant by an increased ability to kill cells and insensitivity to cell survival signals. Thus, phosphorylation of serine 136 and 14-3-3 binding serves as a potent inhibitory mechanism to prevent Bad-induced apoptosis. 14-3-3 can act in a similar fashion to block cell death caused by other death promoters, such as ASK1 (Zhang et al., 1999). By exerting control over multiple proapoptotic proteins, 14-3-3 may function as a general promoter of cell survival.

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