# Mutation of BAD within the BH3 domain impairs its phosphorylation-mediated regulation

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**Abstract Pro-apoptotic functions of the BH3-only protein BAD are negatively regulated by survival signal-mediated phosphorylation at several serine residues. Recently, we found that the mutant BAD (BADD119G) with an amino acid substitution of Asp (Asp119 to Gly) within the BH3 domain displays strong pro-apoptotic activity in serum-starved COS-7 cells, although it cannot interact with Bcl-2. Here, we demonstrate that the BADD119G loses phosphorylation-mediated negative regulation. Importantly, pro-apoptotic activity of wild-type BAD (BADwt) was strongly suppressed by co-transfection with constitutively active Akt (CA-Akt) cDNA, whereas that of BADD119G was not. In these transfectants, BADD119G phosphorylation was barely detectable at serine residues (S75 and S99), although BADwt phosphorylation was clearly increased by CA-Akt. In addition, various external stimuli UV, TPA and forskolin could not phosphorylate BADD119G neither at S75, S99 nor S118 in COS-7 cells. However, in vitro kinase assay revealed that catalytic protein kinase A (PKA) strongly phosphorylated both BADs at S75 and S118, excluding the possibility that the target sequence of PKA was disrupted by mutation at S119. Furthermore, as a result of disrupted phosphorylation, BADD119G could not physically interact with 14-3-3. Taken together, disruption of phosphorylation-mediated negative regulation may explain, at least in part, the strong pro-apoptotic functions of BADD119G, and suggest a role for the BH3 domain in phosphorylation events.**


*Key words:* BAD; BH3 domain; Mutation; PKA; 14-3-3; Phosphorylation; Apoptosis

## 1. Introduction

Bcl-2 family proteins share homologous structures, BH1 to BH4 domains [1,2]. Among these regions, the BH3 domain is responsible for interaction with other Bcl-2 family proteins and plays pivotal roles in regulating apoptosis [3]. Since all BH3-only proteins identified so far – for example, BAD, Bid, Bik, Bim, Bmf, BNIP3, Hrk, and Noxa – can interact with apoptosis-related molecules, and function as pro-apoptotic molecules, the region is believed to be crucial for pro-apoptotic functions. However, there are some data suggesting that the BH3 domain of the pro-apoptotic proteins may not directly serve pro-apoptotic functions. Indeed, functional analysis of a mutant Bik has revealed that hetero-dimerization with survival proteins alone is insufficient for its cell death activity [4]. Moreover, a mutation within the BH3 domain of BAX disrupts its ability to interact with Bcl-2 and Bcl-$X_L$, without disturbing its ability to induce apoptosis [5]. These accumulating data suggest that pro-apoptotic molecules can introduce death signals independently of the BH3-mediated interaction, and the functions of the BH3 domain may vary among the pro-apoptotic proteins.

A BH3-only protein, BAD, is unique, since its functions are tightly regulated by serine phosphorylation [6,7]. Biologically active BAD is a dephosphorylated form and interacts with either Bcl-2 or Bcl-$X_L$ to neutralize their anti-apoptotic functions, and this neutralization is believed to account for its pro-apoptotic 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-$X_L$ [7,8]. Thus, phosphorylation and dephosphorylation of BAD can switch its binding target and determine its pro-apoptotic activity. To date, four serine residues S112, 136, 155 and 170 in murine BAD are known to be phosphorylated in response to survival signals. Phosphorylation of BAD at S112 mainly depends upon a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase-mediated phosphorylation event or its downstream p90Rsk-mediated pathway [9,10]. Phosphorylation of BAD at S136 requires the serine/threonine kinase protein kinase B (Akt) [11,12], while BAD phosphorylation at S155 mainly requires protein kinase A (PKA) [13]. Furthermore, recent studies have revealed a novel BAD phosphorylation site at S170 in cytokine-dependent MC/9 mast cells [14]. Importantly, BAD S170 is phosphorylated in response to GM-CSF stimulation and the phosphorylation correlates well with cell survival, although it remains uncertain which kinase(s) is responsible for the phosphorylation. In this context, phosphorylation of all these serine residues inactivates BAD pro-apoptotic functions.

Recently, we have found that a human BAD mutant (BADD119G) with a single amino acid substitution within the BH3 domain can interact with Bcl-$X_L$, but not with Bcl-2. Interestingly, BADD119G displays a similar pro-apoptotic activity to BADwt in serum-deprived COS-7 cells [15]. These results are consistent with the previous suggestion that the pro-apoptotic actions of BAD are primarily dependent on neutralization of Bcl-$X_L$, and that its neutralization of Bcl-2

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*Abbreviations:* GFP, green fluorescent protein; GST, glutathione *S*-transferase; PKA, protein kinase A; Akt, serine/threonine kinase protein kinase B; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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is marginal [6]. Further characterization of BADD119G prompted us to realize that overexpression of BADD119G somehow induced more extensive apoptosis in COS-7 cells than in BADwt. This suggests that the mutation within the BH3 domain might modulate its pro-apoptotic activity. To explore this possibility, we further investigated BADD119G functions and their regulation. We here demonstrate that BADD119G loses phosphorylation-mediated negative regulation and this may explain, at least in part, its stronger pro-apoptotic activity.

## 2. Materials and methods

### 2.1. Cell culture

Three cell lines, green monkey kidney epithelial COS-7 and human embryonal HEK 293, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C in 5% $CO_2$.

### 2.2. Reagents and antibodies

TPA, forskolin (FK), okadaic acid (OA) and catalytic PKA subunit were purchased from Sigma (St. Louis, Mo, USA). The mammalian expression vectors described below were transfected into cells using LipofectAMINE 2000 Reagent (Gibco Life and Technologies, MD, USA). Antibodies against BAD, phospho-BAD (S112, 136 and 155) and Akt were from Cell Signaling Technology (Beverly, MA, USA). Ser75, 99 and 118 in human BAD are equivalent to Ser112, 136 and 155 in murine BAD, respectively. Antibodies against Hsc70 and hemagglutinin were from Santa Cruz Biotechnology Inc. (CA, USA) and Roche (IN, USA), respectively.

### 2.3. Plasmids

Full-length human BAD cDNA (BADwt was kindly provided by Dr. J.C. Reed of the Burnham Institute) and mutant BADD119G, in which Asp119 was replaced with Gly, was generated as described previously [15]. These cDNAs were sub-cloned into green fluorescence protein (GFP) expression vectors pEGFPs (Clontech, CA, USA). The 14-3-3α cDNA was isolated from human placenta MATCHMAKER cDNA library (Clontech) by a yeast two-hybrid system in our laboratory (unpublished data). These cDNAs were also subcloned into the prokaryote pGEX 4T expression vectors (Amersham Pharmacia Biotech, IL, USA) for the generation of glutathione-*S*-transferase (GST) fusion proteins. Constitutively active Akt (CA-Akt) cDNA expression vector was kindly provided by Dr. M.E. Greenberg (Harvard Medical School).

### 2.4. Apoptosis

To evaluate cell viability, cells were mixed with the same volume of 0.4% trypan blue solution, and immediately examined under the light microscope for dye exclusion. The caspase-3 colorimetric protease assay was performed following the manufacturer's protocol (CPP32/Caspase-3 Colorimetric Protease Assay kit, MBL, Nagoya, Japan). Briefly, $1\times10^6$ cells were lysed in 120 μl of chilled lysis buffer and incubated on ice for 10 min. Total cell lysates were collected and their protein concentration measured using a Protein Assay (Bio-Rad, NY, USA). The lysates (100 μg) were mixed with the same volume of 2× Reaction Buffer, incubated with the 4 mM DEVD-pNA caspase-3 substrate (200 μM final concentration) at 37°C for 2 h, and then analyzed at 400 nm in a spectrophotometer (Shimazu, Tokyo, Japan).

### 2.5. Western blot analysis

The $1\times10^6$ cells were lysed by the addition of 100 μl of lysis buffer containing 100 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1% NP-40 and 50 mM Tris–HCl (pH 7.2). Total cell lysates (80 μg/lane) were separated in 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels and then electrophoretically transferred to PVDF membranes (Millipore, MA, USA) at 20 V for 70 min. Membranes were soaked into 8% BSA (Sigma) overnight at 4°C and washed with washing buffer containing 140 mM NaCl, 25 mM Tris–HCl (pH 7.8) and 0.05% Tween 20. The membranes were incubated with primary antibodies overnight at 4°C and thereafter incubated with the corresponding peroxidase-linked secondary antibodies for 1 h at room temperature. Blots were visualized by an ECL system (Amersham Pharmacia Biotech).

### 2.6. GST fusion protein expression and pull-down assays

pGEX plasmids encoding full-length BADwt, BADD119G, and 14-3-3 as GST fusion proteins were generated as described above. The fusion proteins were induced in BL21-bacteria with isopropyl-β-D-thiogalactoside and were then purified with glutathione–Sepharose 4B (Amersham Pharmacia Biotech). For control experiments GST alone was generated from the pGEX-4T vector. The fusion protein-bound beads were washed and resuspended in 10 mM Tris, pH 7.4, 150 mM NaCl, 5% glycerol, 1% phenylmethylsulfonyl fluoride and 1 μg/ml leupeptin. The concentration of GST fusion protein bound to the beads was estimated visually from Coomassie blue-stained SDS–PAGE with albumin standards. For co-precipitation assays whole cell lysates (1 mg) of HEK 293 transfected cells described above were incubated with 20 μl of GST fusion protein-bound beads for 1–2 h at 4°C. The beads were washed at least five times with ice-cold lysis buffer followed by separation via SDS–PAGE. Proteins were then subjected to Western blot analysis.

### 2.7. In vitro kinase reactions

The in vitro kinase reactions were performed as described previously [16]. To define the ability of BADwt and BADD119G to be phosphorylated, their GST fusion proteins were bound to glutathione–Sepharose beads, washed three times with lysis buffer, twice with kinase buffer (40 mM HEPES [pH 7.4], 3 mM $MnCl_2$, 10 mM

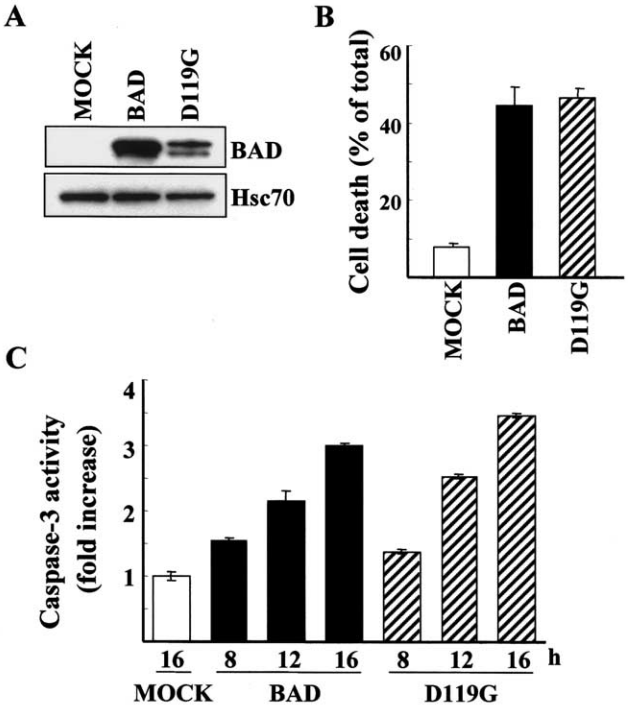


Fig. 1. BAD overexpression induces apoptosis. COS-7 cells were transiently transfected with control pEGFP vector (MOCK), GFP-human BADwt or GFP-BADD119G and cultured in DMEM supplemented with 10% FCS. A: Overexpression of GFP-BAD proteins. 24 h after transfection, cells were harvested and subjected to immunoblot analysis. Hsc70 shows the same amount of protein loaded in each lane. B: BAD overexpression augmented cell death. Cell death was evaluated at 24 h after transfection by trypan blue exclusion assay. C: BAD overexpression increased caspase-3 activity. Transfectants were cultured for 8–16 h, and subjected to caspase-3 measurement. The numbers represent the fold increase in absorbance at 400 nm relative to the mock transfectants harvested at 16 h after transfection. Each column (MOCK, open; BADwt, closed; BADD119G, hatched) displays the mean ± S.D. of data from three separate experiments in panels B and C.

$MgCl_2$ and 1 mM DTT) and resuspended in 30 μl of the kinase buffer. The kinase reaction was initiated by the addition of PKA catalytic subunit (7.5 units) and 100 μM ATP. Following 60 min incubation at 30°C, the reactions were terminated by the addition of SDS loading buffer, and the resultant samples were subjected to SDS–PAGE analysis. BAD phosphorylation was evaluated by antibodies against phospho-BAD (S112 and 155).

## 3. Results

Previously, we demonstrated that both GFP-fused human full-length wild-type BAD (BADwt) and its mutant BADD119G induced apoptosis in serum-starved COS-7 transfectants [15]. To further analyze their biological activity, we first compared their potential pro-apoptotic activity in serum-containing COS-7 cells. Gene transfer of these BAD cDNA expression vectors produced a substantial amount of protein in both transfectants (Fig. 1A). Overexpression of BADwt strongly induced cell death and increased caspase-3 activity in a time-dependent manner in COS-7 transfectants (Fig. 1B, C). Although the expression level of BADD119G was obviously less than that of BADwt, BADD119G showed a similar pro-apoptotic potential to BADwt, as assessed by cell death and activation of caspase-3 (Fig. 1B,C). Caspase-3 activity was slightly higher in BADD119G transfectants at 12–16 h than in BADwt transfectants (Fig. 1C). Thus, there was a slight difference between BADwt and BADD119G transfectants when they were cultured in serum-containing medium. However, a large difference was observed in the apoptosis of COS-7 cells co-transfected with CA-Akt. CA-Akt displayed greater mobility than endogenous Akt due to deletion of its negative-regulatory domain (Fig. 2A). Co-transfection of BADwt with CA-Akt significantly suppressed BADwt-mediated augmentation of cell death and caspase-3 activity (Fig. 2B,C). In contrast, co-transfection with CA-Akt did not suppress BADD119G-mediated augmentation of apoptosis, although CA-Akt expression was greater than in BADwt-co-transfectants. Consideration of previous findings that BAD pro-apoptotic activity is strongly inhibited by Akt activation through its direct phosphorylation of BAD [11,12] and the loss of sensitivity to Akt activation in BADD119G transfectants suggest that BADD119G may abrogate Akt-mediated phosphorylation. We therefore investigated the phosphorylation level of BAD in both CA-Akt-BADwt and CA-Akt-BADD119G co-transfectants. CA-Akt co-transfection clearly increased BADwt phosphorylation levels at S75 and S99 (equivalent to S112 and S136 in murine BAD, respectively), whereas phosphorylation of BADD119G was not visible (Fig. 2A). Even overexpression of CA-Akt failed to phosphorylate BADD119G. This is probably because of insensitivity of BADD119G to CA-Akt. To explore whether this happens in other cells, we investigated the effect of CA-Akt overexpression on BADD119G-mediated apoptosis in NIH3T3 cells. As observed in COS-7 cells, sensitivity of BADD119G to Akt was also impaired (data not shown), suggesting that this defect is a general one regardless of cell type.

We further explored whether BADD119G loses its ability to

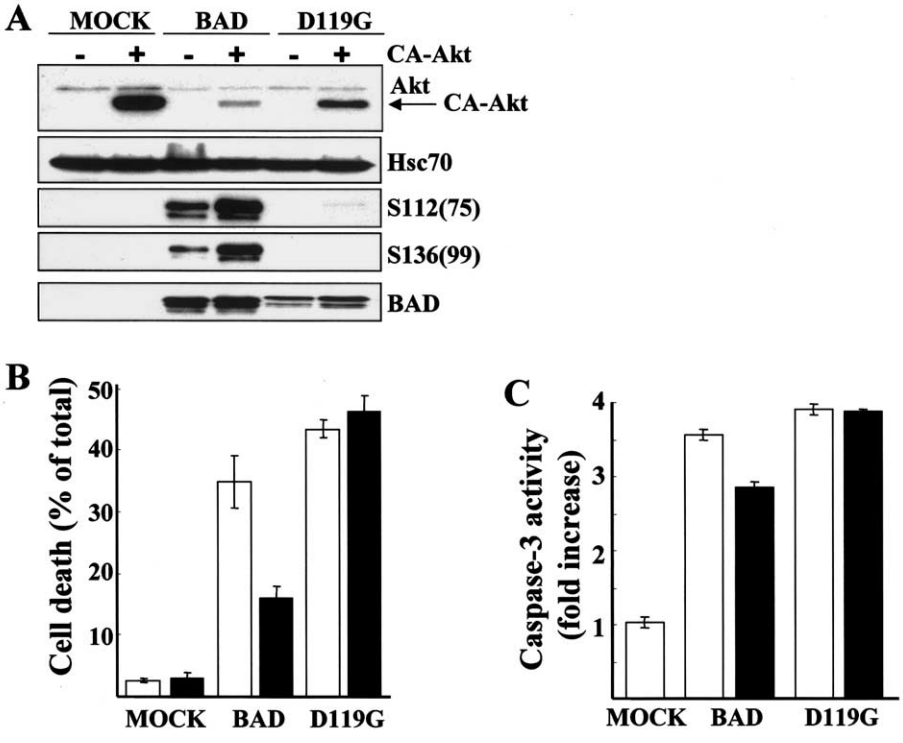


Fig. 2. BADD119G mutant was resistant to CA-Akt overexpression. COS-7 cells were transiently transfected with pEGFP (MOCK), GFP-BADwt (BAD), or GFP-BADD119G (D119G) expression vectors. A: Expression of BAD with CA-Akt proteins and BAD phosphorylation. Transfectants were cultured with FCS, harvested at 21 h after co-transfection either with (+) or without (−) the CA-Akt expression vector and subjected to Western blot analysis. Hsc70 shows the same amount of protein loaded in each lane. B: Effect of CA-Akt on BAD-mediated cell death. At 21 h after transfection, cell death was evaluated by trypan blue exclusion assay. C: Effect of CA-Akt on BAD-induced caspase-3 activation. At 12 h after co-transfection, caspase-3 activity was measured. The numbers represent the fold increase in absorbance at 400 nm relative to the mock transfectants. In panels B and C, COS-7 cells were transfected with the indicated vectors together with CA-Akt (closed columns) or its vehicle (open columns). Each column displays the mean ± S.D. of data from three separate experiments.

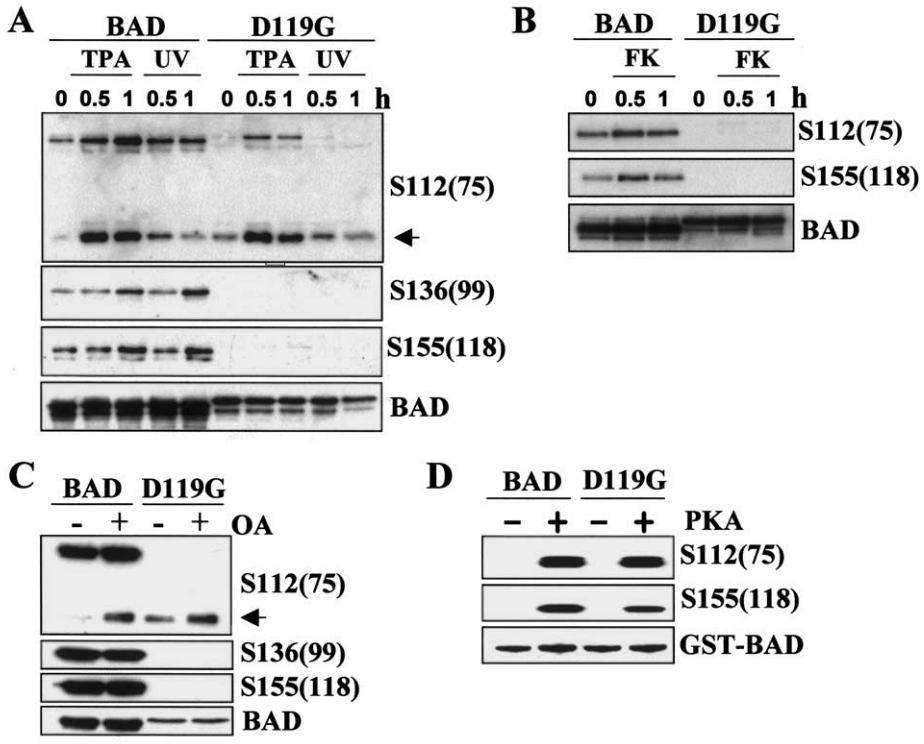


Fig. 3. Phosphorylation of BADwt (BAD) and BADD119G (D119G). A: TPA and UV exposure cannot phosphorylate BADD119G effectively. COS-7 cells were transfected with the indicated expression vectors. After 12 h, cells were deprived of FCS for 10 h, treated with 100 nM TPA or exposed to 40 J/m$^2$ UV and harvested at indicated hours. B: FK treatment cannot phosphorylate BADD119G. After 12 h serum deprivation, COS-7 transfectants were treated with 20 μM FK for the indicated time. C: OA has no significant effect on phosphorylation of BADD119G. At 4 h after transfection, medium was refreshed and COS-7 transfectant cells were further cultured in the presence (+) or absence (−) of 20 nM OA for 14 h. In panels A–C, cells were harvested, and Western blot analysis was carried out using anti-phospho-BAD S112 (75), S136 (99) or S155 (118) antibodies or control BAD antibody. In panels A and C, arrows indicate strong phosphorylation of intrinsic BAD at S112 in COS-7 cells. D: In vitro phosphorylation of GST–BAD proteins. PKA catalytic subunit (7.5 unit) was incubated with GST–BADwt (BAD) or GST–BADD119G (D119G) for 30 min at 25°C. PKA-mediated phosphorylation of these substrates was evaluated by Western blot analysis. Loaded substrates were visualized by separation on 12% SDS–PAGE and staining with Coomassie brilliant blue R-250 (GST–BAD).

be phosphorylated efficiently due to various external stimuli. In COS-7 cells, TPA and UV exposure strongly increased phosphorylation levels of BADwt at S75, S99 and S118 (equivalent to S155 in murine BAD) phosphorylation sites (Fig. 3A). However, these stimuli did not increase phosphorylation levels of BADD119G as seen with BADwt, although TPA faintly increased phosphorylation at S75 only. Importantly, these stimuli clearly phosphorylated intrinsic BAD at S112 even in BADD119G-expressing transfectants as observed in BADwt transfectants (arrow in Fig. 3A). We next investigated the effect of 10 μM FK, which increases cAMP levels and activates PKA, on the phosphorylation of BADs, since PKA is the serine/threonine kinase responsible for S112 and S155 phosphorylation of murine BAD [17–19]. In COS-7 transfectants, FK treatment clearly increased phosphorylation levels of BADwt at S75 and S118, but not those of BADD119G (Fig. 3B). To explore whether the observed lack of phosphorylation of BADD119G in COS-7 cells is general, we further investigated the effect of FK treatment on phosphorylation levels of BADD119G in HeLa transfectant cells. As observed in COS-7 cells, both serines of BADD119G were not phosphorylated by FK as efficiently as BADwt (data not shown). To explore the molecular mechanism(s) by which the BADD119G lost phosphorylation-mediated regulation, we investigated the effect of OA on its phosphorylation levels. OA strongly inhibits PP2A and weakly inhibits PP1, both of which are involved in the dephosphorylation of BAD [20,21]. Although OA treatment clearly increased basal phosphorylation of intrinsic BAD at S112 in COS-7 cells (arrow in Fig. 3C), it did not increase BADD119G phosphorylation (Fig. 3C), making it unlikely that either PP2A or PP1 phosphatase activity contributes to decreased phosphorylation levels in BADD119G. We next investigated whether BADD119G can be phosphorylated by PKA in vitro, since the mutation at S119 may disrupt PKA-mediated phosphorylation at S118. The GST–BADwt and GST–BADD119G fusion proteins [15] were used as exogenous substrates in an in vitro kinase assay with the PKA catalytic subunit. An in vitro kinase assay revealed that both fusion proteins were strongly phosphorylated by catalytic PKA, although S118 of GST–BADD119G was slightly less phosphorylated (Fig. 3D). This strongly suggests that the mutation at S119 did not totally disrupt the ability of S118 to be phosphorylated by PKA. These data indicate that the mutant BADD119G has a defect in its phosphorylation at all three major phosphorylation sites, and probably this defect causes insensitivity to CA-Akt and sustained pro-apoptotic activity.

Phosphorylated BAD is inactivated by its direct interaction with 14-3-3 and their physical interaction prevents BAD from

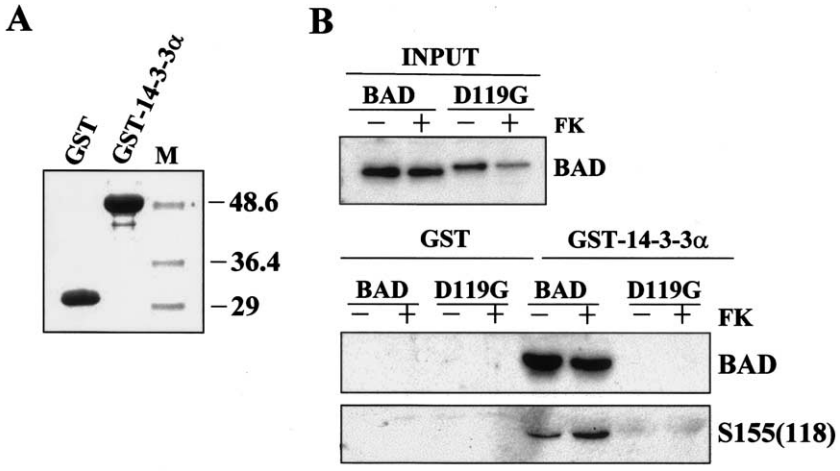


Fig. 4. Interaction of human BAD (BAD) or BADD119G (D119G) with 14-3-3α. A: Expression of GST or GST–14-3-3 fusion proteins. Total GST fusion protein amounts per bead were estimated virtually using Coomassie blue-stained SDS–PAGE. Molecular size marker (M) is shown in kDa. B: Specific binding of BADwt, but not BADD119G, to the GST–14-3-3α fusion proteins. Cell lysates (1 mg) from HEK 293 cells transfected with GFP-tagged BAD or BADD119G expression vector were passed over GST or GST–14-3-3 beads. The bound proteins were separated via SDS–PAGE and identified by Western blots using anti-BAD antibody as a probe. Total proteins loaded are shown in the upper panel (INPUT). To investigate the effect of FK, HEK 293 transfectants were stimulated by 100 nM FK (+) and harvested at 30 min after the treatment.

targeting mitochondria [6,7,22]. It is thus likely that BADD119G cannot interact with 14-3-3. To study this possibility, we carried out a GST pull-down analysis. The GST–14-3-3α fusion proteins were immobilized on glutathione beads (Fig. 4A), and incubated with cell lysates of BADwt or BADD119G-expressing HEK 293 cells, which are resistant to BAD overexpression (data not shown). Western blot analysis revealed the specific pull-down of BADwt, but not BADD119G, by 14-3-3α (Fig. 4B). In addition, the impaired interaction between BADD119G and 14-3-4α could not be restored by FK treatment. Thus, BADD119G lost its ability to interact with 14-3-3 proteins as a result of its impaired phosphorylation.

## 4. Discussion

Here, we demonstrate that a single amino acid mutation of BAD within its BH3 domain impairs its phosphorylation at all major phosphorylation sites and allows its pro-apoptotic activity to persist even in transfectants overexpressing CA-Akt. In this regard, BADD119G is resistant to its inhibitor kinase Akt. The accumulating data showing that Akt is strongly activated in many primary cancer cells [23–25] suggest that BADD119G may introduce apoptotic signals into these carcinomas.

In this study, we found that a single amino acid 119D substitution within the BH3 domain of BAD extensively destroyed its ability to be phosphorylated and to interact with 14-3-3. Indeed, BADD119G is hypo- or un-phosphorylated in COS-7, NIH3T3 and HeLa cells and its phosphorylation is only faintly detectable even after stimulation with UV, TPA and FK treatment. In contrast, substitution of alanine for leucine (L; another well-conserved amino acid residue) within the BH3 domain of BAD strongly reduces its ability to interact with Bcl-$X_L$ or Bcl-2, but increases its interaction with 14-3-3 to a greater extent than BADwt [8]. Together with the data showing its retarded mobility on SDS–PAGE, the authors suggested that the mutated BAD at the L residue may be somehow highly phosphorylated and this hyper-phosphorylation may explain its strong interaction with 14-3-3 and subsequent loss of pro-apoptotic functions. These data suggest that the BH3 domain of BAD is a crucial site, not only for interaction with other Bcl-2 family members, but also for its own phosphorylation machinery.

It remains obscure why the mutant BADD119G loses its ability to be phosphorylated. Our preliminary studies exclude the possibility that increased dephosphorylation is involved in the mechanism, since there was no significant effect of the PP1 inhibitor OA on phosphorylation levels of BADD119G, although OA strongly increased phosphorylation levels of intrinsic BAD at S75. Furthermore, mutation at the D residue did not totally destroy acceptor sites for kinases, since in vitro kinase assay revealed that PKA strongly phosphorylated both BADs (S75 and S118). At this point, we believe that the mutation at the 119D residue within the BH3 domain probably changes its whole BAD conformation and blocks its accessibility to responsible kinases in vivo. In this regard, our study highlights the importance of the three-dimensional conformation of the BH3 domain of BAD. According to its predicted structure, the acidic 119D residue is located at the center of the BH3 domain, and is surrounded by basic residues R and K [8]. We speculate that these charged residues might be crucial for maintenance of its entire BAD conformation.

A recent study has revealed that Cdc2 kinase phosphorylates murine BAD at S128 and inhibits the interaction of BAD with 14-3-3 proteins in cerebellar neurons [26]. This indicates that the novel and distinct BAD phosphorylation at S128 activates its pro-apoptotic functions. In this context, BAD phosphorylation regulates its pro-apoptotic functions either positively or negatively. Interestingly, BADD119G displayed a slower mobility on SDS–PAGE than BADwt. This allows us to consider that BADD119G may be aberrantly phosphorylated and survival signal-mediated phosphorylation may be disrupted at S75, S99 and S118. Disregarding the precise molecular mechanism for un-phosphorylation at these sites of BADD119G, our data indicate that the acidic D res-

idue is crucial for phosphorylation-mediated negative regulation of BAD.

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