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Ca^{2+}-Induced Apoptosis Through Calcineurin Dephosphorylation of BAD

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The Ca^{2+}-activated protein phosphatase calcineurin induces apoptosis, but the mechanism is unknown. Calcineurin was found to dephosphorylate BAD, a pro-apoptotic member of the Bcl-2 family, thus enhancing BAD heterodimerization with Bcl-x_L and promoting apoptosis. The Ca^{2+}-induced dephosphorylation of BAD correlated with its dissociation from 14-3-3 in the cytosol and translocation to mitochondria where Bcl-x_L resides. In hippocampal neurons, L-glutamate, an inducer of Ca^{2+} influx and calcineurin activation, triggered mitochondrial targeting of BAD and apoptosis, which were both suppressible by coexpression of a dominant-inhibitory mutant of calcineurin or pharmacological inhibitors of this phosphatase. Thus, a Ca^{2+}-inducible mechanism for apoptosis induction operates by regulating BAD phosphorylation and localization in cells.

Sustained increases in cytosolic-free Ca^{2+} lead to activation of the serine-threonine phosphatase (PPase) calcineurin (PPase-B) and subsequent apoptosis in susceptible cells (*1*). Overexpression of active calcineurin induces apoptosis through a mechanism that is suppressible by Bcl-2 (*2*). Bcl-2 family proteins regulate a distal step in an evolutionarily conserved pathway controlling apoptosis (*3*). Many Bcl-2 family proteins are anchored in the outer membranes of mitochondria, but oriented toward the cytosol. BAD is a pro-apoptotic member of this family that heterodimerizes with anti-apoptotic proteins such as Bcl-2 and Bcl-x_L, promoting cell death (*4*). Phosphorylation of BAD is induced by growth factors, impairing its binding to Bcl-x_L and abrogating its pro-apoptotic effects in cells (*5*). Several protein kinases can phosphorylate BAD, including Akt (*5*), which mediates cell survival signals within the phosphatidylinositol 3′ kinase pathway (*6*); protein kinase A (PKA) (*7*), a kinase previously implicated in cell survival (*8*); and Raf-1, which promotes cell survival when targeted to mitochondrial membranes through interactions with Bcl-2 (*9*).

We explored the possibility that phosphorylated BAD (phospho-BAD) might be a substrate of calcineurin involved in apoptosis. Transfection of 293T cells with a plasmid encoding a constitutively active form of calcineurin that lacks a negative regulatory domain (ΔCnA/B) (*2*) decreased ^{32}P incorporation into BAD (Fig. 1A). Coexpression of ΔCnA/B with either constitutively active Akt(E40K) (*6, 10*) or mitochondria-targeted active Raf-1 (M-Raf) (*9*) restored ^{32}P labeling of BAD. Experiments with a BAD mutant, BAD(S75E, S99E), in which two serines previously implicated in Akt-mediated phosphorylation (*5, 7, 9*) were replaced with an unphosphorylatable residue (glutamic acid), provided evidence that Akt and Raf-1 target different sites on BAD (Fig. 1B). However, regardless of whether active Akt or Raf-1 was used, coexpression of ΔCnA/B markedly reduced ^{32}P incorporation into BAD (Fig. 1B), suggesting that calcineurin can dephosphorylate multiple sites on this pro-apoptotic protein in cells. Consistent with a direct interaction, ΔCnA/B could be coimmunoprecipitated from lysates of 293T cells transfected with the BAD(S75E, S99E) protein, which mimics a phosphorylated form of BAD recognized as a substrate by this PPase (*11*).

Though a constitutively active form of calcineurin could induce dephosphorylation of BAD both in cells (Fig. 1, A and B) and in vitro (Fig. 1, C to E), we also examined whether endogenous calcineurin could associate with the endogenous BAD protein and control its phosphorylation state in untransfected cells. Calcineurin is the only known serine-threonine PPase whose activity is stimulated by Ca^{2+} (*12*). Changes in BAD phosphorylation were therefore examined in cells treated with Ca^{2+}-mobilizing agents, including the Ca^{2+} ionophores ionomycin and A23187 (*13*) and the endoplasmic reticulum Ca^{2+}–adenosine triphosphatase inhibitor thapsigargin (THG), which induces Ca^{2+} release from internal stores (*14*). HCT116 colon cancer and Du145 prostate cancer cells contain high endogenous phospho-BAD when grown with serum (*15*). Treatment of these cells with Ca^{2+}-mobilizing agents induced dephosphorylation of BAD, as determined by immunoblot analysis with antibodies specific to phospho-BAD, without altering the amount of BAD protein (Fig. 1F) (*13*). Akt kinase activity was unchanged under these same conditions (*13*), suggesting that the reduced amount of phospho-BAD was a result of active dephosphorylation of BAD rather than kinase suppression.

Coimmunoprecipitation experiments revealed that Ca^{2+}-mobilizing agents triggered dissociation of BAD from 14-3-3 (Fig. 1F). Moreover, endogenous calcineurin could be coimmunoprecipated with endogenous BAD before treatment with Ca^{2+}-mobilizing agents. In contrast, PPase-2A was not coimmunoprecipitated with BAD (Fig. 1G), indicating specificity of the calcineurin-BAD interaction. Interestingly, little or no calcineurin complexed with BAD after induction of dephosphorylation with Ca^{2+}-mobilizing agents (Fig. 1F). Thus, calcineurin appears to exist in a complex containing phospho-BAD before, but not after, Ca^{2+}-

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induced activation of this PPase. Similarly, dissociation of BAD-calcineurin complexes was induced by removal of serum growth factors from cultures, which results in reduced activity of endogenous Akt, Raf-1, and other kinases, allowing constitutive PPases to gradually dephosphorylate BAD (Fig. 1F) (*13*).

Coimmunoprecipitation experiments were also performed to determine whether calcineurin-induced changes in BAD phosphorylation correlate with alterations in heterodimerization with Bcl-x_L. In 293 or 293T cells coexpressing FLAG-BAD and Bcl-x_L, ΔCnA/B induced increased association of BAD with Bcl-x_L (Fig. 2A), consistent with its ability to dephosphorylate BAD (Fig. 1). In contrast, Akt(E40K) and M-Raf partially inhibited the ΔCnA/B-induced association of BAD with Bcl-x_L, correlating with their ability to partially restore phosphorylation of BAD (Fig. 1, A and B). Bcl-x_L did not coimmunoprecipitate with BAD(S75E, S99E) protein (Fig. 2A), regardless of whether it was coexpressed with ΔCnA/B (*11*).

The functional relevance of calcineurin-mediated dephosphorylation of BAD was explored by assessing the effects on cell death of coexpressing BAD and ΔCnA/B. Coexpression of BAD and ΔCnA/B greatly increased cell death [measured 2 days later by propidium iodide (PI) exclusion assay] compared with cells expressing either BAD or ΔCnA/B alone (Fig. 2B), demonstrating functional synergy between BAD and this PPase. Nearly all of the PI-positive cells had apoptotic morphology (*11*). Active Akt(E40K) and active M–Raf-1 significantly reduced cell death induction by the combination of BAD and ΔCnA/B, consistent with their ability to restore phosphorylation of BAD (Fig. 1) and to inhibit the effects of ΔCnA/B on BAD–Bcl-x_L dimerization (Fig. 2A). In contrast to the wild-type BAD protein, the BAD (S75E, S99E) mutant failed to synergize with ΔCnA/B in inducing cell death (Fig. 2B), consistent with its inability to dimerize with Bcl-x_L (Fig. 2A) (*16*). The BAD(S75E, S99E) protein also failed to induce apoptosis when expressed alone (*11*).

Transfection of a BAD mutant in which the serines associated with Akt-mediated

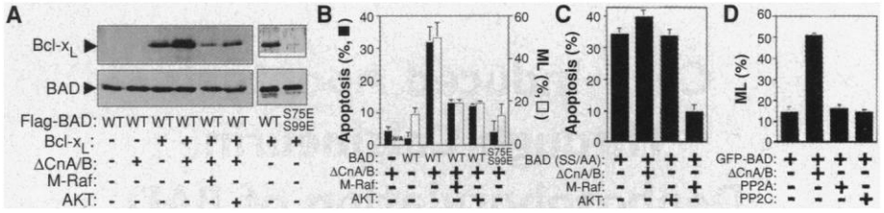

Fig. 2. Calcineurin induces BAD translocation to mitochondria, dimerization with Bcl-x_L, and BAD-mediated apoptosis. (**A**) For coimmunoprecipitation experiments (*30*), 293 cells were transiently transfected with 7 μg of total DNA, including 0.5 μg of pFLAG-CMV2-BAD or pFLAG-CMV2-BAD(S75E, S99E), 0.5 μg of pcDNA3-Bcl-x_L, 1.5 μg each of pMEP plasmids encoding HA-ΔCnA and CnB, 3 μg of M–Raf-1– or HA-Akt(E40K)–producing plasmids, or pcDNA3 plasmid DNA. After 2 days, cell lysates were prepared (*9*) and FLAG-BAD was immunoprecipitated with anti-FLAG followed by immunoblot analysis of the resulting immune-complexes with either anti–Bcl-x_L (top) or anti-BAD (bottom) polyclonal antisera. (**B** and **C**) Cultures of 293 cells were transfected with plasmids encoding wild-type (WT) GFP-BAD, GFP-BAD(S75E, S99E) mutant, or control GFP (*11*) and 1 μg each of pMEP encoding HA-ΔCnA and CnB, 3 μg of M-Raf– or HA-Akt(E40K)–producing plasmid, or parental plasmid DNA. The percentage (≥ 200 cells counted) of dead GFP-positive cells was determined by PI-exclusion assay (mean ± SD; $n = 3$) (black bars) at 2 days and the percentage of cells with punctate fluorescence indicative of mitochondrial or internal membrane localization (ML %) was determined (mean ± SD; $n = 3$) at 1 day (*9*) (N/A, not applicable). For (C) 293 cells were cotransfected with plasmids encoding FLAG–mouse BAD(S112A, S136A), and either ΔCnA/B, Akt(E40K), or M–Raf-1. The percentage of apoptotic cells (mean ± SD; $n = 3$) was determined 2 days later. (**D**) GFP-BAD location was determined as above for cells transfected with plasmids encoding various PPases (*31*) (PP2A, PPase 2A; PP2C, PPase 2C).

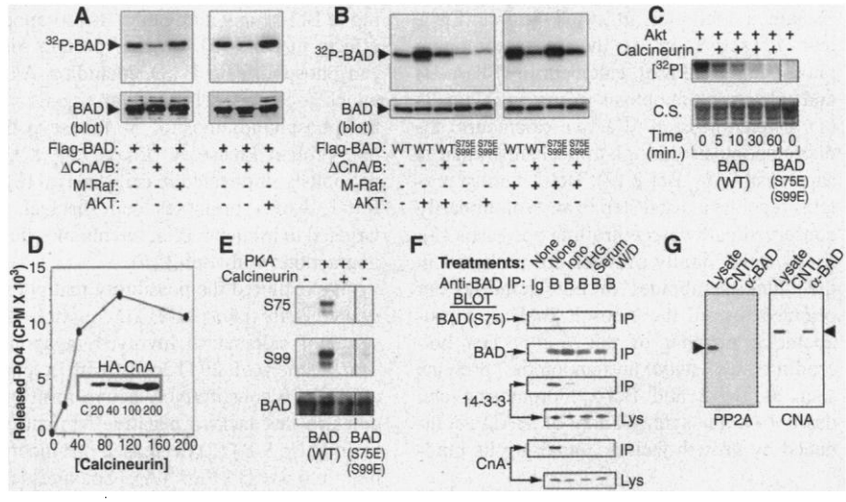

Fig. 1. Calcineurin dephosphorylates BAD protein. (**A** and **B**) Transient transfection of 293T cells (*9*) with various plasmids, as indicated (*27*). Cells were cultured either for 2 days in medium containing 5% (v/v) fetal bovine serum (A) or changed to 0.05% serum after 1 day to minimize growth factor activation of endogenous kinases (B), then labeled with $^{32}PO_4$ for 4 hours (*9*). FLAG-tagged BAD was immunoprecipitated with antibody to FLAG (anti-FLAG), subjected to SDS-PAGE, and transferred to nitrocellulose filters, followed by exposure to x-ray film for detection of ^{32}P-labeled FLAG-BAD (top) (WT, wild type). Blots were subsequently probed with polyclonal rabbit antiserum to BAD (bottom), followed by emission chemiluminescence (ECL)-based detection (*15*). (**C** to **E**) Glutathione S-transferase (GST)–tagged BAD or GST-BAD(S75E, S99E) proteins were phosphorylated in vitro with purified GST-Akt (C), Raf-1 (D), or PKA (E) and either [γ-^{32}P]ATP (C and D) or unlabeled ATP (E) (*28*). After purification with glutathione-Sepharose, phosphorylated GST-fusion proteins were incubated with fixed (C and E) amounts of calcinceurin (2 μg) (*28*) or variable (D) amounts of hemagglutinin (HA)-tagged calcineurin (immunoprecipitated from 0 to 200 μg of cell lysate) (*28*) for variable [0 to 60 min in (C)] or fixed [30 min in (D); 10 min in (E)] times before analysis by SDS-PAGE and autoradiography (C), counting of released ^{32}P (D), or immunoblotting with antibodies specific for phospho-S75 or phospho-S99 in BAD. Coomassie-stained (C.S.) input GST-BAD and GST-BAD(S75E, S99E) proteins are shown (C), as well as immunoblot analysis of input HA-CnA with anti-HA (D) and of GST-BAD with anti-BAD (E). (**F**) HCT116 cells were cultured for 1 day with or without serum and 1 μM THG or ionomycin (Iono), as indicated. Cell lysates were normalized for protein content and anti-BAD (B) immunoprecipitates (IP) were prepared and subjected to SDS-PAGE and immunoblot analysis with antibodies specific for phospho-BAD (S75), total BAD, 14-3-3, or the A subunit of calcineurin (*29*). In some cases, the lysates (Lys) were analyzed directly (15 μg of protein). Control immunoglobulin G1 (IgG1) immunoprecipitations (Ig) were also analyzed for all lysates, confirming specificity, but are only shown for untreated HCT116 cells. (**G**) Endogenous BAD was immunoprecipitated from HCT116 lysates and the immune-complexes were analyzed by immunoblotting with anti-CnA or anti–PPase 2A (CNTL, control; α-BAD, anti–phospho-BAD; PP2A, PPase 2A).

phosphorylation were mutated to alanine, BAD(SS/AA), resulted in far more apoptosis compared with wild-type BAD, consistent with the inability of Akt and some other kinases to phosphorylate this mutant protein and to prevent its dimerization with Bcl-x_L (*5, 7, 9*). In contrast to BAD, however, in cells expressing BAD(SS/AA), active calcineurin only marginally enhanced apoptosis, further confirming the specificity of the functional synergy observed between calcineurin and wild-type BAD (Fig. 2B). As expected (*5, 7, 9*), active Akt was unable to suppress apoptosis induced by BAD(SS/AA) (Fig. 2C). Nevertheless, active M–Raf-1 still suppressed apoptosis induction by BAD(SS/AA), indicating that Raf-1 regulates the activity of BAD through different phosphorylation sites than Akt and some other kinases.

Unlike most Bcl-2 family proteins, BAD has no membrane-anchoring domain (*4*). If calcineurin-mediated dephosphorylation of BAD protein induces dimerization with anti-apoptotic Bcl-2 family proteins, calcineurin should promote association of BAD with intracellular membranes where Bcl-2 and Bcl-x_L reside. To test this hypothesis, we expressed green fluorescent protein (GFP)–tagged BAD in 293 cells, with or without ΔCnA/B. Fluorescence microscopy showed that when cultured with serum, 293 cells expressing GFP-BAD exhibited a mostly diffuse cytosolic pattern. In contrast, when coexpressed with ΔCnA/B, GFP-BAD was concentrated in punctate foci within the cytosol of more than half of the GFP-positive cells (Fig. 2B). Two-color analysis with a mitochondria-specific dye revealed that much of the GFP-BAD protein was associated with mitochondria in these cells (*11*). Overexpression of either active Akt(E40K) or M-Raf partially prevented ΔCnA/B-induced localization of BAD to mitochondria (Fig. 2B). Moreover, the human BAD(S75E, S99E) mutant was resistant to ΔCnA/B-induced localization to mitochondria, consistent with its inability to associate with Bcl-x_L. These effects of ΔCnA/B appeared to be specific, because overexpression of the constitutively active PPase-2A or PPase-2C did not cause relocalization of GFP-BAD (Fig. 2D), but did cause dephosphorylation of several proteins (*11*).

Prostate cancer cells are known for their sensitivity to Ca^{2+}-induced apoptosis (*17*), so we chose them as an additional model for correlating changes in phosphorylation with the intracellular location of BAD by monitoring the endogenous BAD protein rather than relying on GFP tagging. When Du145 cells were cultured in serum-containing medium, the BAD protein was phosphorylated (*13*) and was located diffusely throughout the cytosol, as determined by indirect immunofluorescence microscopy (Fig. 3A). In contrast, treating Du145 cells with THG or A23187 resulted in BAD dephosphorylation (*13*) and relocalization of BAD to mitochondria (Fig. 3, B and C). Targeting of BAD to mitochondria was evident within 1 hour and maximal within 4 to 6 hours, preceding apoptosis, which was maximal at ~1 day after THG or A23187 treatment.

Calcineurin has been implicated in neuronal cell death induced by insults that elevate cytosolic Ca^{2+} (*18, 19*), prompting us to examine the location of BAD in neuronal apoptosis models. Immortalized rat hippocampal CSM14.1 cells transfected with GFP-BAD and treated with THG (Fig. 3, E and F) or A23187 (*11*) showed a redistribution of GFP-BAD protein from the cytosol to mitochondria and other internal membranes. In contrast, cotransfection of a trans-dominant inhibitory mutant of CnA/B, ΔCnA(H101Q), prevented the THG-induced redistribution of GFP-BAD in these cells (Fig. 3G) and reduced apoptosis from 71% ± 5% to 30% ± 4%. The GFP-BAD(S75E, S99E) protein failed to translocate from the cytosol to membranes, consistent with its inability to dimerize with Bcl-x_L (Fig. 3H). In contrast to the ΔCnA(H101Q) dominant-inhibitory protein, RNA and protein synthesis inhibitors did not prevent Ca^{2+}-induced apoptosis of CSM14.1 cells, indicating that gene transcription is not required (*11*).

We obtained similar results with primary rat hippocampal neurons, using 1 mM L-glutamate as a stimulus for inducing increases in cytosolic Ca^{2+} (*20*). L-Glutamate induced the translocation of GFP-BAD (Fig. 3, J to L) but not GFP (*13*) from cytosol to mitochondria. In contrast, coexpression of the inhibitor, ΔCnA(H101Q), prevented L-glutamate–induced redistribution of GFP-BAD in most of these cells (Fig. 3, K and L) and completely suppressed L-glutamate–induced

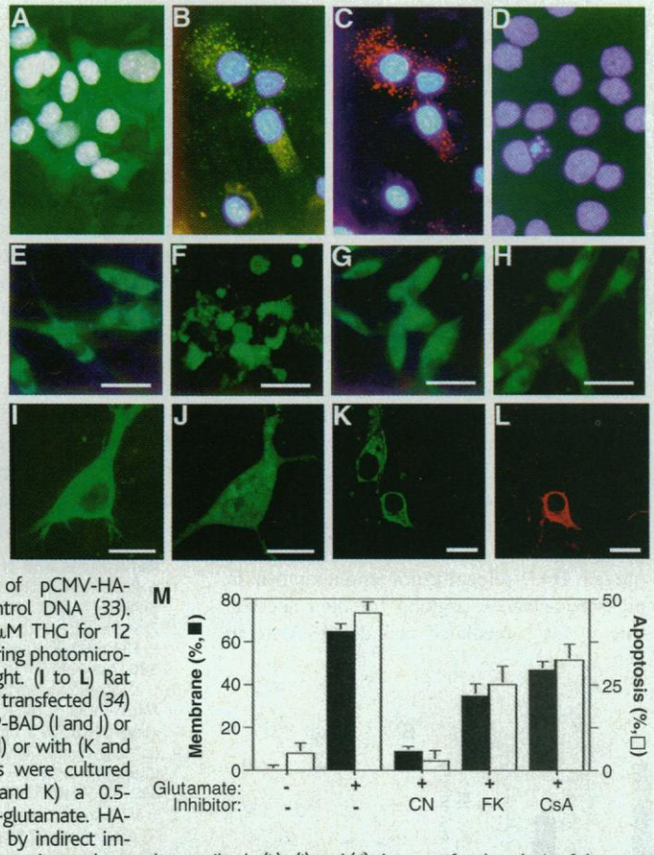

Fig. 3. Ca^{2+}-mobilizing agents induce calcineurin-dependent translocation of BAD from to mitochondria and apoptosis. (**A** to **D**): Du145 cells were cultured without (A) or with 1 μM THG (B to D) for 0.5 day, when most of the cells remained viable. Cells were fixed and stained with antibodies to BAD (A and B), mitochondrial Hsp60 (C), or IgG1 control (D), followed by either FITC-conjugated (A, B, and D) or rhodamine-conjugated (C) secondary antibodies (*32*). (**E** to **H**) CSM14.1 cells were transfected with 1 μg of pEGFP-BAD (E to G) or pEGFP-BAD (S75E, S99E) (H) and either 3 μg of pCMV-HA-ΔCnA(H101Q) (G) or control DNA (*33*). Cells were exposed to 1 μM THG for 12 hours (F to H) before preparing photomicrographs under ultraviolet light. (**I** to **L**) Rat hippocampal neurons were transfected (*34*) with plasmids encoding GFP-BAD (I and J) or GFP control (*13*), without (I) or with (K and L) HA-CnA (H101Q). Cells were cultured without (I) or with (J and K) a 0.5-hour exposure to 1 mM L-glutamate. HA-CnA(H101Q) was detected by indirect immunofluorescence with Cy3-conjugated secondary antibody (L). (I) and (J) show confocal analysis of the same GFP-BAD–expressing neuron before and at 4 hours after L-glutamate treatment (bar, 10 μm). (K) and (L) show a field from a culture of L-glutamate–treated cells containing two neurons successfully transfected with GFP-BAD plasmid, only one of which expresses HA-CnA(H101Q) (bar, 20 μm). Note that GFP-BAD is associated with mitochondria in the neuron that failed to express HA-CnA(H101Q), producing ringlike green emissions from the surface of these organelles. In contrast, GFP-BAD is excluded from the mitochondria (appear as holes in the cytosol) of the L-glutamate–treated neuron that expresses HA-CnA(H101Q). Locations of mitochondria were confirmed by preculturing cells with MitoTracker (*13*). (**M**) Hippocampal neurons transfected with plasmids encoding GFP-BAD, CnA(H101Q) (CN), or various combinations were cultured with or without 1 mM L-glutamate. In some cases, cells were pretreated for 0.5 hour with 0.5 μM CsA or FK506. The percentages of cells with membrane-associated GFP-BAD (membrane %) at 6 hours (closed bars) and apoptotic nuclear morphology (based on DAPI staining) at 24 hours (open bars) were determined (mean ± SE; $n = 3$) (*35*).

apoptosis under these in vitro conditions (Fig. 3M). Immunolocalization studies of another calcineurin substrate, NF-AT (nuclear factor of activated T cells), revealed that L-glutamate–induced translocation of NF-AT from cytosol to nucleus in >95% of control neurons compared with <5% of neurons expressing ΔCnA(H101Q), thus providing further evidence that the calcineurin dominant-inhibitory mutant was effectively suppressing endogenous calcineurin function (*13*).

FK506 (0.5 μM) and cyclosporin A (CsA) (0.5 μM), drugs that indirectly inhibit calcineurin through their effects on petidyl-proyl-cis/trans-isomerases (*1*), were equally potent as ΔCnA(H101Q) at suppressing L-glutamate–induced nuclear translocation of NF-AT (*13*), but less effective at inhibiting mitochondrial targeting of GFP-BAD (Fig. 3M). The extent of FK506- and CsA-mediated suppression of GFP-BAD relocalization, however, correlated well with apoptosis suppression ($P < 0.05$) (Fig. 3M), implying that phospho-BAD rather than phospho–NF-AT is a more relevant substrate of calcineurin under these circumstances and suggesting that FK506-FKBP and CsA-cyclophilin drug-protein complexes preferentially interfere with calcineurin's interactions with phospho–NF-AT compared with phospho-BAD. Of note, although CsA can have cytoprotective effects beyond calcineurin suppression due to its binding to mitochondrial cyclophilin and interference with mitochondrial permeability transition pore opening (*21*), the effects of FK506 and ΔCn(H101Q) cannot be explained by this alternative mechanism. Moreover, FK506 and ΔCn(H101Q) were consistently more potent than CsA at suppressing L-glutamate–induced translocation of GFP-BAD and apoptosis in neurons (Fig. 3M).

These findings obtained by monitoring the translocation of GFP-BAD in transfected neurons were verified to also apply for the endogenous BAD protein in untransfected hippocampal neurons. Localization of endogenous BAD protein by immunofluorescence microscopy revealed diffuse cytosolic staining in untreated cells, whereas L-glutamate exposure caused mitochondrial targeting of BAD (Fig. 4A) (*22*). Furthermore, immunostaining with an antibody to phospho-BAD provided evidence that L-glutamate treatment is associated with dephosphorylation of endogenous BAD protein in hippocampal neurons, because untreated neurons were nearly all immunopositive whereas about half of L-glutamate–exposed cells became immunonegative (Fig. 4A). In contrast, addition of FK506 to neuronal cultures reduced the percentage of L-glutamate–treated cells with mitochondria-targeted BAD, decreased L-glutamate–induced loss of anti–phospho-BAD antibody reactivity, and diminished apoptosis of these untransfected neurons (Fig. 4A). These L-glutamate–induced changes in BAD localization and anti–phospho-BAD antibody reactivity were associated with increased binding of endogenous BAD to Bcl-x_L, and with slightly decreased association of BAD with endogenous calcineurin, as determined by co-immunoprecipitation experiments (Fig. 4B). FK506 diminished L-glutamate–induced formation of BAD–Bcl-x_L dimers, consistent with involvement of calcineurin (Fig. 4B).

Inhibition of BAD targeting to mitochondria and apoptosis by ΔCn(H101Q) in hippocampal neurons was specific to circumstances where calcineurin activation is known to occur. For example, when 1 μM staurosporine was used as a general inhibitor of kinases necessary for cell survival, GFP-BAD relocalized to mitochondria and apoptosis was induced regardless of whether cells were cotransfected with CnA(H101Q)-producing plasmids or treated with FK506 or CsA (*13*). Thus, calcineurin-independent mechanisms for controlling BAD phosphorylation and mitochondrial targeting also exist; calcineurin inhibitors do not prevent BAD relocalization and apoptosis in all circumstances, arguing for their specific role in Ca^{2+}-mediated cell death. Also, in contrast to 1 mM L-glutamate, cytotoxicity induced by a high dose (10 mM) of L-glutamate was not suppressible by CnA (H101Q) (*13*), suggesting that alternative Ca^{2+}-initiated cell death pathways can be activated under such conditions (*18, 21, 23*).

Though pathological elevations in cytosolic Ca^{2+} concentrations can induce apoptotic or necrotic cell death through multiple mechanisms, the finding that redistribution of BAD and apoptosis were suppressible by an inhibitory mutant of calcineurin argues that this PPase is a significant mediator of cell death signals in at least some cellular contexts. Calcineurin has been implicated in both transcription-dependent and -independent apoptosis, with the former attributed to calcineurin-mediated dephosphorylation of NF-AT and subsequent trans-activation of apoptosis genes such as Fas-ligand (*24*). Our results suggest that calcineurin can also induce transcription-independent apoptosis by dephosphorylating BAD, thus allowing the BAD protein to dimerize with Bcl-x_L or other Bcl-2 family proteins located in mitochondrial and other internal membranes. Thus, the interaction of calcineurin with BAD provides a Ca^{2+}-inducible mechanism for controlling the phosphorylation state and hence the bioactivity of this pro-apoptotic protein, thereby linking calcineurin to the apoptosis machinery. The relative ineffectiveness of FK506 and CsA at suppressing BAD targeting to mitochondria and apoptosis compared with ΔCnA(H101Q) illustrates the need for active-site inhibitors of calcineurin that directly interfere with its PPase activity for neuronal protection. Conversely, suppression of calcineurin-mediated dephosphorylation of BAD conceivably could contribute to immune system–independent cancer progression induced by CsA (*25*).

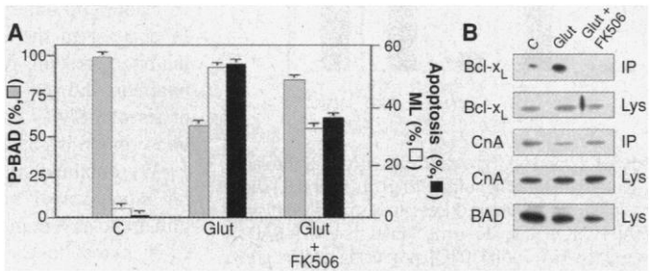

Fig. 4. L-Glutamate induces dephosphorylation, translocation, and Bcl-x_L binding of endogenous BAD in neurons. Hippocampal neurons were cultured without (C) or with 1 mM L-glutamate (Glut), 0.5 μM FK506, or both reagents. (**A**) Neurons were fixed and confocal immunofluorescence was performed with either anti–phospho-BAD(S112) (gray bars) or anti-BAD (*15*) (open bars), counting cells ($n \geq 100$) with phospho-BAD immunopositivity (%P-BAD) or with BAD translocation to mitochondria (ML%) (*22*) at 6 hours. Apoptosis was determined at 1 day by DAPI staining (mean ± SD; $n = 3$). (**B**) Lysates from cultured neurons were used for immunoprecipitation (IP) of BAD with a monoclonal antibody (Transduction Labs), followed by SDS-PAGE and immunoblot analysis with anti-BAD, anti–Bcl-x_L, or anti-CnA. Lysates (25 μg of total protein) were also analyzed directly for comparison of amounts of input proteins (ECL detection).

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27. In Fig. 1A, a total of 8.5 μg of DNA was transfected in 100-mm dishes, including 0.5 μg of pFLAG-CMV2-BAD and 2.5 μg each of pMEP encoding HA epitope–tagged ΔCnA [a Ca^{2+}- and calmodulin-independent form of the A subunit of calcineurin (*2*)] and the B subunit (CnB), and either 3 μg of M-Raf– (*9*) or HA-Akt(E40K)–producing (*6*) plasmids, or parental plasmid DNA as a control (designated by "−" in the figure). In Fig. 1B, 293 cells were transiently transfected with a total of 5.5 μg of DNA, including 0.5 μg of pFLAG-CMV2 encoding wild-type BAD or BAD (S75E, S99E) mutant, 1 μg of pcDNA3-M-Raf-1 or pCMV6-HA-Akt(E40K), and 2 μg each of pMEP encoding HA-tagged ΔCnA and CnB, or parental plasmid DNA (−). The mutants BAD(S75E, S99E) and Akt(E40K) were generated by polymerase chain reaction mutagenesis with pFLAG-CMV2-BAD as the template to substitute Ser^{75}(TCC)/Ser^{99}(TCG) for Glu^{75}(GAA)/Glu^{99}(GAG) or pCMV6-HA-AKT, respectively. Plasmids encoding GFP-BAD and GFP-BAD(S75E, S99E) were generated by cloning these BAD cDNAs into pEGFP-C2 (Clontech) with Eco RI and Xho I sites.
28. For in vitro dephosphorylation reactions, recombinant baculovirus GST-Akt was purified from NP-40 Sf9 cell lysates with GSH-agarose (Sigma). A 1-μg sample of GST-huBad or GST-huBad(S75E, S99E) (hu designates human protein) was phosphorylated in vitro at 30°C for 1 hour. The kinase reaction was stopped by NP-40 lysis buffer, and GST-containing proteins were recovered with GSH-agarose. The precipitates were washed in NP-40 lysis buffer, phosphate-buffered saline (PBS), and calcineurin phosphatase buffer [50 mM tris (pH 7), 0.1 mM $CaCl_2$, 2 mM $NiCl_2$, bovine serum albumin (0.25 mg/ml), calmodulin (0.02 mg/ml; Boehringer-Mannheim). The precipitates were resuspended in 100 μl of phosphatase buffer, and the reaction was started by addition of 2 μg of calcineurin (Upstate Biotechnology, Lake Placid, NY). Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by autoradiography. Alternatively, GST-BAD immobilized on glutathione-Sepharose was ^{32}P-labeled with baculovirus-produced Raf-1 (*9*), then treated with various amounts of immunoprecipitates, which were prepared from 293T cells transiently transfected with HA-CnA/B (20 to 200 μg of total protein lysate) or pMEP control ("C") plasmid (200 μg of total protein lysate), respectively, with anti-HA. Release of ^{32}P label from GST-BAD was measured after 0.5 hour at 30°C. Anti-HA immunoblot analysis was performed to quantify input HA-ΔCnA/B used for the in vitro dephosphorylation reactions. For PKA, recombinant GST-huBAD or GST-huBAD(S75E, S99E) (1 μg) was incubated with 1 mU of PKA (Boehringer-Mannheim) in 40 μl of PKA kinase buffer (40 mM tris-HCl, 20 mM $MgCl_2$, pH 7.4) containing 1 mM dithiothreitol and 1 mM adenosine triphosphate (ATP) at 30°C for 1 hour. Dephosphorylation reactions were carried out at 37°C with 2 μg of calcineurin. Samples were analyzed by SDS-PAGE and immunoblotting with anti-phospho-mBAD(S112) [New England Bio Labs (NEBL)] (serine residue S112 in mouse BAD corresponds to S75 in human BAD) and anti-phospho-mBAD(S136) (corresponding to S99 in human BAD). Total BAD protein was assessed using an anti-BAD monoclonal antibody (*15*), and ECL detection.
29. Cell lysates (*9*) were subjected to immunoprecipitation with monoclonal anti-BAD (*15*) immobilized on protein G–Sepharose. Proteins were separated by SDS-PAGE and analyzed by immunoblotting with antiserum to BAD anti-S112 mBAD (NEBL), anti–14-3-3 (Santa Cruz Biotechnologies), or anti-CnA (Chemicon).
30. Two days after transfection 293 cells were lysed in 0.5 ml of NP-40 lysis buffer (137 mM NaCl, 20 mM tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.2% NP-40) containing protease and phosphatase inhibitors (*9*). Lysates were mixed with 15 μl of anti-FLAG M2 affinity gel for 3 hours at 4°C. The beads were washed three times with 1 ml of NP-40 lysis buffer supplemented with protease and phosphatase inhibitors, boiled in Laemmli buffer, and the eluted proteins were subjected to 12% SDS-PAGE and immunoblot analysis with rabbit antisera specific for Bcl-x_L or BAD (*15*).
31. We transiently transfected 293 cells with normalized total amounts of plasmid DNA, including 1 μg of pEGFP-BAD or pEGFP-BAD(S75E, S99E), 1 μg each of HA-ΔCnA– and CnB-expressing plasmids, 1 μg of PPase 2A– or PPase 2C–producing plasmids, 3 μg of M-Raf or HA-Akt (E40K), or various combinations of these as indicated. The location of GFP was determined by confocal microscopy (*9*). In some experiments, mitochondria labeling was performed by addition of 50 nM Mitotracker (Molecular Probes) to culture medium for 0.5 hour.
32. For immunofluorescence, cells were fixed in 4% paraformaldehyde/PBS (pH 7.4) for 0.5 hour and blocked in 5% (v/v) goat serum. After permeabilization in 0.2% Triton X-100 for 10 min, cells were incubated with polyclonal rabbit anti-HA or anti-Hsp60 (1:500; Berkeley Antibody), which were detected with Cy3- or rhodamine-conjugated goat anti–rabbit IgG (1:100; Chemicon) or with anti-BAD (*15*) followed by fluorescein isothiocyanate (FITC)–conjugated rabbit anti-mouse IgG (1:100; DAKO), and analyzed with a Bio-Rad MRC 1024 confocal laser scanning microscope.
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34. Rat E17 hippocampal neuronal cultures free of glia were prepared as described (*26*). The hippocampal neurons were transiently transfected by a calcium phosphate precipitation method (MBS transfection kit, Stratagene) (*26*) and exposed to 1 mM L-glutamate 3 days later for 0.5 hour, followed by incubation in DMEM for an additional 4 to 24 hours in 5% CO_2/10% O_2 at 37°C.
35. Comparisons were also performed of neurons that were cultured in insulin-containing medium for 3 days, then either exposed for 0.5 hour to 1 mM L-glutamate before they were returned to usual culture conditions or exposed continuously to 10 μM staurosporine (*13*). Replicate cultures of cells were fixed at either 6 hours for analysis of GFP-BAD (direct visualization) and myc–NF-AT (anti-myc indirect immunofluorescence) location or at 24 hours for assessment of apoptosis by staining with 4′6′-diamidino-2-phenylindole (DAPI) (10 μg/ml).

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