14-3-3 Interacts Directly with and Negatively Regulates Pro-apoptotic Bax*

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The Bcl-2 family of proteins comprises well characterized regulators of apoptosis, consisting of anti-apoptotic members and pro-apoptotic members. Pro-apoptotic members possessing BH1, BH2, and BH3 domains (such as Bax and Bak) act as a gateway for a variety of apoptotic signals. Bax is normally localized to the cytoplasm in an inactive form. In response to apoptotic stimuli, Bax translocates to the mitochondria and undergoes oligomerization to induce the release of apoptogenic factors such as cytochrome c, but it is still largely unknown how the mitochondrial translocation and pro-apoptotic activity of Bax is regulated. Here we report that cytoplasmic protein 14-3-3 binds to Bax and, upon apoptotic stimulation, releases Bax by a caspase-independent mechanism, as well as through direct cleavage of 14-3-3 by caspases. Unlike Bad, the interaction with 14-3-3 is not dependent on the phosphorylation of Bax. In isolated mitochondria, we found that 14-3-3 inhibited the integration of Bax and Bax-induced cytochrome c release. Bax-induced apoptosis was inhibited by overexpression of either 14-3-3 or its mutant (which lacked the ability to bind to various phosphorylated targets but still bound to Bax), whereas overexpression of 14-3-3 was unable to inhibit apoptosis induced by a Bax mutant that did not bind to 14-3-3. These findings indicate that 14-3-3 plays a crucial role in negatively regulating the activity of Bax.

Regulation of programmed cell death, or apoptosis, is essential for normal development and for the maintenance of homeostasis in most metazoans. Various apoptotic signals eventually converge into a common death mechanism, in which members of the cysteine protease family (known as caspases) are activated and cleave various cellular proteins. In mammals, the mitochondria play an essential role in apoptosis by releasing apoptogenic factors such as cytochrome c, Smac/Diablo, and Omi/HtrA2 from the intermembrane space into the cytoplasm (1–3). Once in the cytoplasm, cytochrome c binds to Apaf-1, a mammalian homologue of Ced-4, that recruits and activates initiator caspase-9, which subsequently activates effector caspase-3/caspase -7 (4, 5), whereas Smac/Diablo and Omi/HtrA2 facilitate caspase activation by interacting with and inhibiting IAPs, the endogenous caspase inhibitor family (3, 6, 7).

The Bcl-2 family of proteins includes the best characterized regulators of apoptosis, comprising anti-apoptotic members, including Bcl-2 and Bcl-xL, and pro-apoptotic members that include multi-domain Bax and Bak and various single-domain BH3-only proteins (1, 8). Proteins of this family directly regulate the release of mitochondrial apoptogenic factors. Many of the pro-apoptotic family members, such as Bax, Bid, Bad, Bim, and Bmf, are localized in the cytoplasm, and apoptotic stimulation results in their translocation to the mitochondria and induction of the release of apoptogenic factors, probably by inactivating anti-apoptotic members of the family and activating multi-domain members like Bax and Bak (9–12). Translocation of the BH3-only proteins appears to involve various post-translational modifications. For instance, cytoplasmic Bid is cleaved by caspase-8 and then undergoes translocation to the mitochondria (13, 14). Dephosphorylation by calcineurin frees Bad from cytosolic 14-3-3 and allows it undergo translocation to the mitochondria (15). Although the mechanism involved is still unknown, Bim and Bmf are freed from microtubular dynein motor complexes and myosin V actin motor complexes, respectively, during certain forms of apoptosis (16, 17). Bax has also been shown to undergo translocation and integration into the mitochondrial membrane during apoptosis (18–22), and the translocation process has been suggested to involve a conformational change of the Bax molecule, especially exposure of the C terminus (20, 21, 22). It has also been reported that translocation of Bax to the mitochondria is enhanced by caspases (20) or by intracellular alkalization (24) and is negatively regulated by Bcl-2 through a still unidentified mechanism (19, 21, 22).

The 14-3-3 proteins (seven isomers in mammals; β, γ, ε, η, σ, πθ, and ζ) are highly conserved cytoplasmic molecules that form homodimers and heterodimers and interact with various cellular proteins. These proteins seem to control various cellular processes by sequestering regulatory molecules (25). The 14-3-3 proteins have also been implicated in signaling for apoptosis through interaction with apoptotic molecules such as Bad (26), ASK1 (27), and FKHR1 (28). Furthermore, 14-3-3e and ζ are known to act as mitochondrial import stimulation factors (29) and appear to play a crucial role in intracellular protein trafficking, although the precise mechanism by which these isomers of 14-3-3 participate in protein translocation is not yet understood.

In the present study, we showed that 14-3-3 protein was bound to Bax in the cytoplasm of living cells and that Bax...
underwent dissociation from this protein by caspase-independent and -dependent mechanisms during apoptosis to induce apoptotic changes of the mitochondria, indicating that 14-3-3ζ plays a crucial role in the negative regulation of Bax activity in living cells.

EXPERIMENTAL PROCEDURES

Antibodies and Chemicals—An anti-human Bax (N20) polyclonal antibody that cross-reacted with mouse Bax was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and an anti-human Bax (Ab-3) monoclonal antibody (mAb) was obtained from Oncogene (Darmstadt, Germany). Anti-heat shock 14-3-3e (T16, cross-reacting with human 14-3-3ζ), anti-rat 14-3-3ζ (C17, cross-reacting with human and mouse 14-3-3ζ), anti-human 14-3-3ζ (C16), and an anti-human 14-3-3ζ mAb (H8) that recognized the N-terminal region of various 14-3-3 family members were purchased from Santa Cruz Biotechnology. An anti-mouse 14-3-3ζ mAb, an anti-Xpress mAb, and an anti-FLAG M2 mAb were purchased from Calbiochem (La Jolla, CA), Invitrogen, and Sigma, respectively. An anti-cterm underlined cysteine c mAb (65881A) that cross-reacted with human cysteochrome c was obtained from Pharmingen (San Diego, CA). Anti-Fas antibody (CH-11) was purchased from MBL (Nagoya, Japan). The cleavable protein cross-linker dimethyl-3,3′-dithiobispropionimidate-2HCl (DTBP) was purchased from Pierce, [32P]orthophosphate was obtained from Amersham Biosciences, and zVAD-fmk was purchased from Enzo Life Sciences. Other chemicals were purchased from Wako Co. (Tokyo, Japan).

Construction of Plasmid Vectors—DNA encoding human 14-3-3ζ, ζ, and various 14-3-3ζ mutants (K49E/V176D, D239A, α1–6, and 1-239 with amino acid residues 1–161, 1–239, respectively) were generated by the PCR using proofreading Pfu DNA polymerase (Strategene, CA) and were subcloned into a recombinant protein-producing vector (pGEX-1T) and two mammalian expression vectors, pUC-CAGGS (30) and pcDNA1.1 (Invitrogen), with an N-terminal His tag and an anti-Xpress epitope. DNA encoding HA-tagged mouse Bax and its mutants (ΔN, Δ1, ΔB3, Δ5/6, and ΔC, lacking amino acid residues 1–20, 22–37, 63–72, 106–153, and 172–193, respectively) were generated by PCR and were subcloned into pBluescript SK (+) (Strategene, CA). DNA encoding FLAG-tagged human Bad was subcloned into pUC-CAGGS.

Protein Purification—Recombinant His-tagged human Bax was expressed and purified as described previously (31). Recombinant human 14-3-3ζ, ζ, and ζ were expressed as GST fusion proteins in Escherichia coli (strain DH5α) and were purified on a glutathione-Sepharose column. 14-3-3ζ was released from GST by cleavage with thrombin and purified to homogeneity by MonoQ chromatography. Mock proteins were produced by the same method using empty plasmids. In some experiments, GST-14-3-3ζ was used without cleavage of GST.

Mock proteins were incubated with 14-3-3ζ and then were centrifuged at 200,000×g for 45 min to separate the mitochondria and unbroken cells by centrifugation at 600×g for 10 min, the post-crude nuclear supernatant was centrifuged at 10,000×g for 10 min to collect the heavy membrane fraction.

Assessment of the Binding of Bax into the Mitochondrial Membrane—Heavy membrane fractions enriched for mitochondria from cells or isolated rat mitochondria were incubated in 0.1 M Na2CO3 (pH 11.5), and then were centrifuged at 200,000×g for 45 min to separate the supernatant and pellet as described elsewhere (21).

Analysis of Bax Translocation and Cytochrome c Release in Vitro—Mitochondria were prepared from the livers of male SD rats in MT-A buffer (0.3 m mannitol, 10 mM potassium Hepes, pH 7.4, 0.1% fatty acid-free bovine serum albumin), as described previously (33). Recombinant 14-3-3ζ (the indicated amounts) and rBax (1 μg) were preincubated for 30 min at 25°C and then were added to the mitochondria (100 μg) and incubated for a further 3 min at 25°C in 100 μl of Mt-B buffer (Mt-A buffer plus 100 μM potassium phosphate and 4.3 mM succinate). Next, the mixture was then centrifuged to collect the mitochondria, and aliquots of mitochondrial or supernatant were analyzed by Western blotting using anti-Bax antibodies. To detect the release of cytochrome c, mitochondria were centrifuged, and the supernatant was analyzed by Western blotting using anti-cytochrome c antibodies.

In Vitro Assay of the Dissociation of Bax from 14-3-3ζ—GST-14-3-3ζ (2 μg) and rBax (5 μg) were preincubated for 8 h at 4°C in pH 7.5 buffer, and then the mixture was incubated with glutathione-Sepharose for 3 h. After brief centrifugation, the beads that bound GST-14-3-3ζ and GST-14-3-3ζ-Bax complex were incubated with either cytoxin (10 μg) in pH 7.5 buffer for 12 h at 25°C, with 200 μl of pH 6.5, pH 7.5, or pH 8.0 buffer (50 mM Tris/His, pH 6.5, 7.5 or 8.0, 0.2% Nonidot P-40) for 12 h at 4°C, or with caspase-8 (600 units) for 4 h at 37°C. After brief centrifugation, the beads were washed and resuspended in the sample buffer and then analyzed by Western blotting.

Analysis of Protein-Protein Interaction—For immunoprecipitation experiments, HeLa and NIH3T3 cells were incubated with 2 μl DTBP (a protein cross-linker) for 30 min. Then the cells were lysed and sonicated in lysis buffer (50 mM Tris/His, pH 7.4, 142.5 mM KC1, 5 mM MgCl2, 1 mM EGTA, and 0.2% Nonidot P-40) containing proteinase inhibitors. To investigate the interaction between exogenous Bax and Bad, 293T cells were transiently transfected with the expression plasmids using LiPOFectAMINE in the presence of zVAD-fmk (100 μM) to prevent Bax- or Bad-induced apoptosis. Then the cells were lysed, and the lysates were subjected to immunoprecipitation with the indicated antibodies, and the precipitates were analyzed by Western blotting. To detect binding between purified proteins, recombinant proteins were incubated for 8 h with either GST-14-3-3 proteins or GST alone in 100 μl of the lysis buffer, and then these proteins were incubated with glutathione-Sepharose for 3 h. After brief centrifugation, the beads were washed and resuspended in the SDS-PAGE sample buffer, as described elsewhere (32). The proteins were analyzed by Western blotting and autoradiography.

Surface Plasmon Resonance—The affinity between Bax and 14-3-3ζ proteins was measured by surface plasmon resonance using a BiaCore2000 (Biacore). Equivalent molar amounts of GST-mock and GST-14-3-3 proteins were immobilized on the sensor chip (CM5; Pharmacia Corp.) by the amine-coupling method. Bax was added as the aniline, and the affinity was calculated from the difference between the resonance units with GST-14-3-3 proteins and those with GST-mock.

Metabolic Labeling—293T cells were transfected with pUC-CAGGS-human Bax DNA or pUC-CAGGS-human Bax DNA using LipofectAMINE (Life Technologies, Inc.), according to the supplier’s protocol. The transfected cells were labeled for 24 h in phosphate-free RPMI 1640 medium with [32P]orthophosphate (4 μCi/106 cells).

Cell Fractionation—Cell fractionation was performed using digitonin, as described previously (22). Briefly, after washing twice with phosphate-buffered saline, the cultured cells were collected and treated with 10 μg digitonin for 5 min at 37°C. The cytosolic and organelar fractions were separated by centrifugation and lysed with RIPA buffer. As a result, more than 92% of cytosolic protein was recovered in the supernatant, and more than 95% of mitochondrial protein was localized to the pellet. The heavy membrane fraction enriched for mitochondria was collected as follows. The post-crude nuclear supernatant was resuspended in phosphate-buffered saline, resuspended in isotonic buffer (20 mM potassium Hepes, pH 7.4, 1.5 mM MgCl2, 100 mM KCl, and 250 mM sucrose), and then homogenized with a Dounce homogenizer. After separation of nuclei and unbroken cells by centrifugation at 600×g for 10 min, the post-crude nuclear supernatant was centrifuged at 10,000×g for 10 min to collect the heavy membrane fraction.

Analysis of Cell Death—293T cells were transiently transfected with human Bax DNA (0.2 μg) with or without DNA expressing human β-galactosidase.
14-3-3 or its mutants (0.5 μg), plus 0.1 μg of the green fluorescent protein (GFP) expression construct (pEGFP-N1; Clontech). Transfected cells were incubated for 24 h at 37°C and stained with 1 μM Hoechst 33342, after which the extent of apoptosis was calculated as the percentage of GFP-positive cells showing nuclear fragmentation relative to all GFP-positive cells.

RESULTS

Bax Interacts with 14-3-3—Although translocation and integration of cytoplasmic Bax into the mitochondrial membrane is a critical step for its pro-apoptotic activity, the mechanism of action is poorly understood. To improve our understanding of the regulation of Bax, we searched for a molecule that interacted with Bax, modulated its activity, and found that Bax was bound to protein 14-3-3 in HeLa cells (Fig. 1a). The same interaction between Bax and 14-3-3 was also observed in NIH3T3 cells (Fig. 1b). Because there are several isoforms of 14-3-3 (25), we next tested the interaction of Bax with other 14-3-3 isoforms. As shown in Fig. 1a, Bax was also bound to 14-3-3 in healthy cells (10 μg of HeLa cell lysate was incubated with 2 μg of the indicated GST-14-3-3 proteins or the equivalent amount of GST-mock protein for 8 h. Then GST-Sepharose was added for 3 h and collected by centrifugation, after which bound rBax was analyzed by Western blotting. Total indicates the total amount of rBax used. d, the indicated amount (15 μg or 30 μg) of rBax or bovine serum albumin was run over a chip containing immobilized GST-mock protein, GST-14-3-3, ζ, 1, or 6, and protein interactions were measured by surface plasmon resonance as described under "Experimental Procedures." e, the amount of each 14-3-3 isoform in HeLa cells. Lysates from healthy HeLa cells (10 μg) and the indicated GST-14-3-3 isoforms (25 ng each) were analyzed by Western blotting using antibodies specific for 14-3-3 (left panel), 14-3-3 (middle panel), and 14-3-3 (right panel). The amount of each of the 14-3-3 isoforms in 10 μg of lysate was estimated by comparison with the GST-14-3-3 proteins using densitometric analysis and is shown below the blots (in nanograms).

14-3-3 or its mutants (0.5 μg), plus 0.1 μg of the green fluorescent protein (GFP) expression construct (pEGFP-N1; Clontech). Transfected cells were incubated for 24 h at 37°C and stained with 1 μM Hoechst 33342, after which the extent of apoptosis was calculated as the percentage of GFP-positive cells showing nuclear fragmentation relative to all GFP-positive cells.

FIG. 2. Regions of Bax essential for interaction with 14-3-3. The indicated mutants of mouse Bax were produced by in vitro translation in the presence of [35S]methionine and were incubated with GST-14-3-3 or GST-mock protein. After GST-14-3-3 and GST-mock protein were precipitated with GST-Sepharose as described under "Experimental Procedures," Bax bound to GST-14-3-3 or GST-mock was analyzed by SDS-PAGE followed by autoradiography. A diagram of the Bax deletion mutants is also shown. These deletion mutants retained the regions shown by horizontal lines. a1–a7 indicate the possible helices retained by Bax.
interaction with 14-3-3. As shown in Fig. 3a, although Bax was bound to 14-3-3, we could not detect any phosphorylation of Bax in 293T cells when overexpressed Bax was labeled with [32P]orthophosphate, a result consistent with previous reports (38). Under the same experimental conditions, we readily detected phosphorylation of Bad (Fig. 3a), which is known to be phosphorylated before binding to 14-3-3 (26). Furthermore, the immunoprecipitated Bad did not react with antibodies specific for phosphoserine or phosphothreonine (data not shown). These results indicated that phosphorylation of Bax was not necessary for interaction with 14-3-3. To further confirm that phosphorylation of Bax did not play an essential role in the interaction with 14-3-3, we examined the binding of Bax to a mutant of 14-3-3 (K49E/V176D) that had lost the ability to bind to various target phosphoproteins, including Raf-1 and ASK1 (27). As shown in Fig. 3b, whereas wild-type 14-3-3 was co-immunoprecipitated with both Bax and FLAG-Bad, 14-3-3/K49E/V176D was co-immunoprecipitated with Bax but not with FLAG-Bad, supporting our hypothesis that phosphorylation of Bax was not necessary for interaction with 14-3-3.

Bax Is Negatively Regulated by 14-3-3 and Dissociates during Apoptosis in Both a Caspase-dependent and Caspase-independent Manner. To obtain some insight into the biological significance of the interaction of Bax with 14-3-3, we next examined whether this interaction was altered during the apoptotic process. As shown in Fig. 4a, treatment with VP16 (etoposide) caused the amount of 14-3-3 interacting with Bax to decrease markedly, and a large fraction of Bax was translo-
activation, dissociation of Bax from 14-3-3-ϕ was only partly inhibited (Fig. 4a), indicating that dissociation occurred in both a caspase-independent and caspase-dependent manner. As shown in Fig. 4c, integration of Bax into the mitochondrial membrane was also partly inhibited by zVAD-fmk, whereas translocation of Bax to the mitochondria was not affected by this caspase inhibitor (Fig. 4b). Note that the amount of caspase-independent dissociation of Bax-14-3-3-ϕ was well correlated with the caspase-independent mitochondrial integration of Bax (Fig. 4, a and c).

The findings obtained using cell lysates were similar to those obtained with living cells. As shown in Fig. 4d, lysates from VP16-treated cells were more efficient at causing Bax to dissociate from 14-3-3-ϕ than lysates from normal cells. Interestingly, this dissociation was partially inhibited in the presence of the caspase inhibitor zVAD-fmk, indicating that dissociation of Bax from 14-3-3-ϕ occurred via both caspase-dependent and -independent mechanisms in the cell lysates (Fig. 4d), as it did in living cells (Fig. 4a). All of these results suggested that 14-3-3-ϕ had a role in the sequestration of Bax.

The dissociation of Bax from 14-3-3-ϕ during apoptosis suggested that 14-3-3-ϕ was a negative regulator of Bax. To test this possibility, we examined whether 14-3-3-ϕ affected the mitochondrial translocation of Bax using isolated mitochondria. As shown in Fig. 5, the addition of recombinant 14-3-3-ϕ (or ϕ) protein inhibited the integration of Bax into the mitochondrial membrane (Fig. 5a) as well as Bax-induced release of cytochrome c (Fig. 5b).

Caspases Directly Cleave 14-3-3-ϕ To Release Bax—To investigate the mechanism of the caspase-dependent dissociation of Bax from 14-3-3-ϕ, we examined whether caspases were able to cleave 14-3-3-ϕ. As shown in Fig. 6a, 14-3-3-ϕ and 14-3-3-ε, but not 14-3-3-ζ, were cleaved during apoptosis, and their cleavage was completely inhibited by the caspase inhibitor zVAD-fmk. When recombinant caspase-3 was added to normal cell lysates, 14-3-3-ϕ was cleaved in a z-VAD-fmk-sensitive manner (Fig. 6b). Furthermore, recombinant caspase-3 also cleaved recombinant 14-3-3-ϕ (Fig. 6c), indicating that 14-3-3-ϕ is a direct target of caspase-3. As shown in Fig. 6c, 14-3-3-ϕ was also cleaved by caspase-7 and caspase-8. In contrast, 14-3-3-ϕ was cleaved by caspase-3 in the presence of cell lysate but not in its absence (Fig. 6, b and c), indicating that 14-3-3-ϕ is not a direct target of caspases, unlike 14-3-3-ζ. To identify the caspase cleavage site in 14-3-3-ϕ, various Asp to Ala mutants were produced. Among them, only the D239A mutant was not cleaved during apoptosis, indicating that Asp\(^{239}\) (not present in 14-3-3-ε and 14-3-3-ζ) is the caspase cleavage site.

As shown in Fig. 6e, when 14-3-3-ϕ was cleaved during apoptosis, cleaved 14-3-3-ϕ was not co-immunoprecipitated with Bax, suggesting that cleaved 14-3-3-ϕ has a decreased affinity for Bax. In fact, full-length r14-3-3-ϕ interacted with rBax, whereas this interaction was greatly diminished when r14-3-3-ϕ was cleaved by caspase-3 (Fig. 6e). Furthermore, when the complex formed by GST-14-3-3-ϕ and rBax was treated with caspase-8, rBax was released, and this release was completely inhibited by a caspase inhibitor (Fig. 6f). These results indicated that caspase-dependent dissociation of Bax from 14-3-3-ϕ could be ascribed to cleavage of 14-3-3-ϕ by caspases.

Bax Dissociates from 14-3-3-ϕ under Basic and Acidic Conditions—It has been demonstrated that in the early phase of apoptosis induced by a variety of stimuli, including cytokine deprivation, cytoplasmic alkalization occurs and induces a conformational change of Bax that results in its integration into the mitochondria (24). Therefore, we examined whether alkali-
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FIG. 7. Caspase-independent dissociation of Bax from 14-3-3. 
(a), decrease of Bax binding to 14-3-3 by treatment at pH 8.0. Upper panel, the interaction between rBax and GST-14-3-3 at pH 7.5 or 8.0 was analyzed using surface plasmon resonance as in Fig. 1d. Lower panel, GST-14-3-3 (2 μg) and rBax (5 μg) were preincubated for 30 min at 4 °C at the indicated initial pH and then were mixed for 8 h at 4 °C in buffer at the indicated terminal pH. The mixture was then incubated with glutathione-Sepharose for 3 h. After a brief centrifugation, the beads were washed, and the amount of rBax and 14-3-3 on the beads was analyzed by Western blotting. Total indicates the total amount of rBax used. 
(b), the pH-dependent dissociation of Bax from 14-3-3. GST-14-3-3 (2 μg) and rBax (5 μg) were preincubated at 4 °C for 8 h, and then the mixture was incubated with glutathione-Sepharose for 3 h. After brief centrifugation, the beads were treated in buffer (pH 7.5, pH 8.0, or pH 6.5 (b) at 4 °C for 12 h. The amount of rHis-Bax and 14-3-3 on the beads was then analyzed by Western blotting.

FIG. 5. Involvement of 14-3-3 in the regulation of apoptosis. 
a, inhibition of Bax-induced apoptosis by co-transfection of 14-3-3. 293T cells were transfected with pUC-CAGGS-human 14-3-3Δ or its mutant (0.5 μg), with or without pUC-CAGGS-HA-tagged Bax (0.2 μg) or a Bax mutant (ΔN), together with the GFP expression construct (0.1 μg) (to identify DNA-transfected cells). After 24 h, apoptosis was assessed from the nuclear morphology by Hoechst 33342 staining of GFP-positive cells. The data are expressed as the means ± S.D. (n = 4). 
b, prevention of Bax translocation to the mitochondria by expression of 14-3-3. A similar experiment to that described in a was conducted. DNA-transfected cells were fractionated, and the extent of Bax translocation to the mitochondria was assessed by Western blotting using anti-Bax antibody. 
c, efficient translocation of BaxΔN to the mitochondria. 293T cells were transfected with pUC-CAGGS-HA-tagged Bax (0.2 μg) or a Bax mutant (ΔN) and then were fractionated. The extent of Bax translocation to the mitochondria was assessed by Western blotting using anti-HA antibody. 
d, prevention of Bax-mediated apoptosis by a caspase-noncleavable mutant of 14-3-3. HeLa cells were transfected with pUC-CAGGS-human 14-3-3Δ or its mutant (0.4 μg) together with the GFP expression construct (0.1 μg) (to identify DNA-transfected cells). After 24 h, the cells were treated with 0.25 μg/ml of anti-Fas antibody (CH-11), and apoptosis was assessed after another 12 h from the nuclear morphology by Hoechst 33342 staining of GFP-positive cells. The data are expressed as the means ± S.D. (n = 4).

14-3-3 Inhibits Bax- and Fas-induced Apoptosis—Finally, we examined the physiological role of the interaction between 14-3-3 and Bax in the regulation of apoptosis. If 14-3-3 negatively regulates Bax, overexpression of 14-3-3 could be expected to inhibit Bax-induced apoptosis. As shown in Fig. 8, (a, left panel, and b), apoptosis induced by transfection of Bax DNA was significantly reduced by co-transfection of 14-3-3Δ DNA, and integration of Bax into the mitochondrial membrane was also inhibited. Importantly, a mutant form of 14-3-3Δ (K49E/V176D) that bound to Bax but not to phosphorylated targets, including Bad (Fig. 3b), also inhibited Bax-induced apoptosis (Fig. 8a, left panel), suggesting that the inhibition was due to direct association with Bax and not to the influence of various other 14-3-3-binding proteins, including Bad, Raf-1, and forkhead protein. Furthermore, a caspase-cleaved mutant of 14-3-3Δ (1–239) with a weak affinity for Bax (Fig. 6f) caused less inhibition of Bax-induced apoptosis, whereas another mutant (14-3-3Δα1–6) that did not bind to Bax (data not shown) could not inhibit such apoptosis (Fig. 8a, middle panel). Incomplete suppression of Bax-induced apoptosis by overexpression of 14-3-3Δ was probably due to the abundance of endogenous 14-3-3Δ. Moreover, as shown in Fig. 8a (right panel), 14-3-3Δ did not inhibit apoptosis induced by BaxΔN, to which it did not bind (Fig. 2). Consistent with the inability of 14-3-3Δ to sequester BaxΔN, we found that BaxΔN showed efficient translocation to the mitochondria (Fig. 8c) and induced more apoptosis than wild-type Bax (Fig. 8a). These results suggested that 14-3-3 inhibits Bax-induced apoptosis in an interaction-dependent manner.

Next, we examined whether overexpression of 14-3-3 could inhibit apoptosis induced by an anti-Fas antibody. Because we found that caspase-8 cleaved 14-3-3 to release Bax, we also tested a caspase-resistant (noncleavable) mutant of 14-3-3Δ (14-3-3ΔD239A). As shown in Fig. 8d, overexpression of 14-3-3ΔD239A significantly inhibited apoptosis induced by anti-Fas antibody, suggesting that the cleavage of 14-3-3 by caspases could facilitate Fas-induced apoptosis.

DISCUSSION

Bax is mainly found in cytoplasmic and/or perimitochondrial locations in living cells, and apoptotic stimulation causes its stable integration into the mitochondrial membrane, along with the induction of cytochrome c release (18–22, 31, 40, 41). However, it is still poorly understood how Bax remains inactive in healthy cells. Although it has been suggested that Bax exists as a monomer in the cytoplasm of healthy cells and forms dimers or oligomers on the mitochondrial membrane during apoptosis (10, 21), the present study clearly showed that a significant fraction of Bax interacts with 14-3-3 in living cells and that this interaction negatively regulates Bax by seques-
tering it to the cytoplasm. Among seven isoforms, 14-3-3e, 14-3-3c (this study), and 14-3-3σ (34) also bind to Bax and probably play a redundant role.

It has been suggested that Bax undergoes a conformational change during apoptosis, on the basis of its increased susceptibility to proteolytic cleavage (20) and binding with some antibodies (42). These changes can be explained by our proposal that Bax dissociates from 14-3-3 during apoptosis. Our finding that both N- and C-terminal regions of Bax were required for its interaction with 14-3-3σ is also consistent with previous observations that translocation of Bax to the mitochondria is stimulated by N-terminal deletion (Fig. 8c and Ref. 20) as well as by mutation of charged residues in the N- and C-terminal regions (23, 24). We also showed that Bax dissociates from 14-3-3σ under both alkaline and acidic conditions, which is consistent with the previous observation that translocation of Bax to the mitochondria is enhanced at an alkaline pH (24).

Taken together, it seems likely that a significant fraction of Bax is sequestered by cytoplasmic 14-3-3 in healthy cells, whereas apoptotic stimuli cause the dissociation of Bax from 14-3-3 and translocation to the mitochondria.

The 14-3-3 proteins are highly conserved cytoplasmic molecules that interact with various cellular proteins and that are thought to be involved in the regulation of various cellular processes, including apoptotic signal transduction (25). It has been reported that different isoforms of 14-3-3 sequester different pro-apoptotic molecules through a phosphorylated serine or threonine residue (25, 36). In contrast, we found that Bax dissociates from 14-3-3σ under both alkaline and acidic conditions, which is consistent with the previous observation that translocation of Bax to the mitochondria is enhanced at an alkaline pH (24).

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