Protein Phosphatase 2A Enhances the Proapoptotic Function of Bax through Dephosphorylation*

Meiguo Xin and Xingming Deng

From the University of Florida Shands Cancer Center and Departments of Medicine and Anatomy & Cell Biology, University of Florida, Gainesville, Florida 32610-0232

Bax is a major proapoptotic member of the Bcl2 family that is required for apoptotic cell death. We have recently discovered that Bax phosphorylation at serine 184 induced by nicotine through activation of protein kinase AKT abolishes its proapoptotic function in human lung cancer cells. Here we found that either treatment of cells with the protein phosphatase 2A (PP2A) inhibitor okadaic acid or specific disruption of PP2A activity by expression of SV40 small tumor antigen enhanced Bax phosphorylation, whereas C2-ceramide, a potent PP2A activator, reduced nicotine-induced Bax phosphorylation, suggesting that PP2A may function as a physiological Bax regulatory phosphatase. PP2A co-localized and interacted with Bax. Purified, active PP2A directly dephosphorylated Bax in vitro. Overexpression of the PP2A catalytic subunit (PP2A/C) suppressed nicotine-stimulated Bax phosphorylation in association with increased apoptotic cell death. By contrast, depletion of PP2A/C by RNA interference enhanced Bax phosphorylation and prolonged cell survival. Mechanistically C2-ceramide-induced Bax dephosphorylation caused a conformational change by exposure of the 6A7 epitope (amino acids 13–19) that is normally hidden at its N terminus that promoted the insertion of Bax into mitochondrial membranes and formation of Bax oligomers leading to cytochrome c release and apoptosis. In addition, PP2A directly disrupted the Bcl2/Bax association to liberate Bax from the heterodimer complex. Thus, PP2A may function as a physiological Bax regulatory phosphatase that not only dephosphorylates Bax but also activates its proapoptotic function.

Apoptosis occurs by activation of an intrinsic or extrinsic pathway and is largely regulated by the Bcl-2 family of apoptotic regulators that is comprised of three subfamilies (1–2). The subfamily including Bcl2, Bcl-XL, and MCL1 members block apoptosis, whereas the Bax subfamily, consisting of Bax and Bak, or the BH3-only subfamily, including Bad, Bid, Bok, Bik, Bim, and PUMA, promote apoptosis (3–8). Bcl2 family members function in a tightly regulated network that protects or induces mitochondrial dysfunction. It is popularly held that antiapoptotic Bcl2 and Bcl-XL heterodimerize with proapoptotic Bax or Bak such that the hydrophobic crevices on their surfaces bind to the exposed BH3 domain of Bax or Bak to block their proapoptotic function. Thus, heterodimerization appears to regulate, at least in part, cell survival or death (5). Furthermore the BH3 domain of the proapoptotic members is required for both their oligomerization and killing activity, although homodimerization does not necessarily correlate with killing activity (9). Genetic studies using Bax and Bak single and double homozgyous knock-out mice reveal that either Bax or Bak is essential for inducing mitochondrial dysfunction characterized by the release of potent caspase activators including cytochrome c (Cyt c) and Smac/Diablo that initiate the intrinsic pathway (10).

Bax is the major proapoptotic Bcl2 family protein that is widely expressed in various human lung cancer cells including both small cell lung cancer and non-small cell lung cancer cells. Recent reports indicate that phosphorylation, a post-translational modification, can regulate the proapoptotic activity of Bax (11–13). The growth factor granulocyte/macrophage colony-stimulating factor and nicotine have been found to induce Bax phosphorylation at Ser-184 through a physiological Bax kinase AKT, which is associated with inactivation of the proapoptotic function of Bax and prolonged cell survival (11, 12). However, whether a physiological phosphatase is involved in dephosphorylation of Bax remains unclear. Phosphatase 2A (PP2A) is a major protein serine/threonine phosphatase that participates in many signaling pathways in mammalian cells (14). It is a heterotrimer consisting of a 36-kDa catalytic subunit (PP2A/C), a 65-kDa structural A subunit (PP2A/A), and a variable regulatory subunit (PP2A/B, which can vary in size from 50 to 130 kDa). The AC catalytic complex alone has phosphatase activity, whereas the distinct B subunits can recruit PP2A/C to distinct subcellular locations and then define a specific substrate target (15–17). The A and C subunits are evolutionary conserved and ubiquitously expressed (12). These two subunits form a catalytic complex (PP2A/A/C) that interacts with at least three families of regulatory subunits (B, B’, and B”) and tumor antigens (i.e. SV40 small tumor antigen; Ref. 14). The B subunits determine the substrate specificity of PP2A (15). There is evidence that the regulatory B subunits may target the catalytic complex to intracellular sites such as microtubules (16), the nucleus, and cytoplasm (17).

Ceramide is a naturally occurring membrane sphingolipid that functions as a critical second messenger molecule in apoptotic cell death signaling (19–22). An association between the production of ceramide and the onset of apoptosis has been well established (23). A number of diverse apoptosis-promoting agents, including tumor necrosis factor α (TNFα; Ref. 23), chemotherapeutic drugs (24, 25), Fas antigen activation (26), irradiation (27), and corticosteroids (28), can all apparently generate ceramide by the induction of sphingomyelin hydrolysis. This indicates that the production of ceramide in response to apoptotic stress stimuli may be a universal element of apoptosis (22). Recent studies show that treatment of cells with water-soluble analogs of ceramide such as C2-ceramide potently induces apoptotic cell death (29, 30). Ceramide may have multiple targets in regulating apoptotic cell death, one
PP2A Dephosphorylates Bax

of which is PP2A because ceramide is able to stimulate PP2A activity through binding of its catalytic subunit (31). Intriguingly the ceramide-activated PP2A is able to dephosphorylate and inactivate mitochondrial Bcl2 in association with increased apoptotic cell death (29). The C2-ceramide mediates dephosphorylation of Bcl2 following translocation of PP2A to the mitochondria (30). Because Bax is a phosphorylatable protein in the Bcl2 family, it is possible that PP2A may also function as a physiological Bax phosphatase to dephosphorylate Bax and regulate its proapoptotic activity. Here we experimentally tested this hypothesis.

EXPERIMENTAL PROCEDURES

Materials—Nicotine and cisplatin were purchased from Sigma. Oka- daic acid, C2-ceramide, and purified PP2A were obtained from Calbio- chem. AKT and phosphospecific AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA). PP2A/C siRNA, anti- Bax, anti-SV40 small tumor (small t) antigen, FITC-conjugated anti- mouse IgG, and rhodamine-conjugated anti-rabbit IgG antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Purified anti-Bax monoclonal 6A7 antibody was purchased from BD Pharmingen. Cross-linkers bis(sulfosuccinimidyl) suberate and disuc- cinimidyl suberate were purchased from Pierce. The HA-tagged PP2A/C/pCDNA3 construct was generously provided by Dr. Brian Law (University of Florida). Wild type small t antigen cDNA in pCMV5 was kindly provided by Dr. Marc Mumby (University of Texas Southwestern Medical Center, Dallas, TX). All reagents used were obtained from commercial sources unless otherwise stated.

Cell Culture—A549 cells were cultured in F-12K medium with 10% fetal bovine serum and 4 mM L-glutamine. H69, H82, H23, H358, H157, H460, and H1299 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum.

Metabolic Labeling, Immunoprecipitation, and Western Blot Analysis—Cells were washed with phosphate-free RPMI 1640 medium and metabolically labeled with [32P]orthophosphoric acid for 60 min. After treatment of cells with an agonist or inhibitor for various times, cells were washed with ice-cold 1× PBS and lysed in detergent buffer. Bax was immunoprecipi- tated using an agarose-conjugated Bax antibody. The samples were sub- jected to 12% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at −80 °C. Bax phosphorylation was deter- mined by autoradiography. The same filter was then probed by Western blot using a Bax antibody and developed using an ECL kit from Amersham Biosciences as described previously (11).

Dephosphorylation of Bax in Vitro—A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. 32P-Labeled Bax was immunoprecipitated using an agarose-conju- gated Bax antibody. The beads were washed three times in detergent buffer and resuspended in 60 μl of phosphate assay buffer containing 50 mM Tris-HCl, pH 7.0, 20 mM β-mercaptoethanol, 2 mM MnCl2, 0.1% bovine serum albumin. Purified PP2A (50 ng) was added, and the sam- ples were incubated at 30 °C for various times as described previously (32). The reaction was terminated by the addition of 2× SDS sample buffer. The sample was boiled for 5 min before loading onto an SDS- polyacrylamide gel. Bax phosphorylation was determined by autoradiography.

Immunofluorescence—A549 cells were seeded and cultured on a Lab- Tek® chamber slide (Nalge Nunc International) overnight at 37 °C with 5% CO2. Cells were washed with 1× PBS, fixed with ice-cold methanol and acetone, and blocked with 10% mouse and rabbit serum. Then cells were incubated with mouse anti-human Bax and rabbit anti-human PP2A/C primary antibodies for 90 min. After washing, samples were incubated with FITC-conjugated anti-mouse and rhodamine-conju- gated anti-rabbit secondary antibodies for 60 min. Cells were washed with 1× PBS and observed under a fluorescence microscope (Zeiss). Pictures were taken and colored with the same exposure setting for each experiment. To determine subcellular regions of protein co- localization, individual red- and green-stained images derived from the same field were merged using Openlab 3.1.5 software from Improvision, Inc. (Lexington, MA). Areas of protein co-localization appear yellow.

Subcellular Fractionation—Cells (2 × 107) were washed with cold 1× PBS and resuspended in isotonic mitochondrial buffer (210 mM mannito- tol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes, pH 7.5) containing protease inhibitor mixture set I (Calbiochem), homogenized with a Polytron homogenizer operating for four bursts of 10 s each at a setting of 5, and then centrifuged at 2000 × g for 3 min to pellet the nuclei and unbroken cells. The supernatant was centrifuged at 13,000 × g for 10 min to pellet mitochondria as described previously (11). The second supernatant was further centrifuged at 150,000 × g to pellet light mem- branes. The resulting supernatant is the cytosolic fraction. Mitochon- dria were washed with mitochondrial buffer twice, resuspended in 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × g for 10 min at 4 °C. The resulting supernatant containing mitochondrial proteins was collected. Protein (100 μg) from each frac- tion was subjected to SDS-PAGE. Bax was analyzed by Western blot using a Bax antibody. The purity of each fraction was confirmed by assessing localization of the mitochondria-specific protein prohibitin (33).

Alkali Extraction of Bax, Which Is Peripherally Associated with Mitochon- drial Membranes—Cells were treated with C2-ceramide (30 μM) for various times. Mitochondria were isolated by subcellular fractionation, resuspended in freshly prepared 0.1 M Na2CO3, pH 11.5, and incubated on ice for 30 min. The samples were then centrifuged at 200,000 × g for 30 min, and the alkali-extracted membrane pellet was resuspended with 1% Nonidet P-40 lysis buffer, rocked for 60 min, and then centrifuged at 17,530 × g for 10 min at 4 °C. The supernatant containing the nonextractable mitochondrial proteins was collected and subjected to SDS-PAGE. The alkali-resistant Bax (i.e. nonextract- able or integral) was determined by Western blot using a Bax antibody as described previously (34, 35).

Cross-linking of Bax in Mitochondrial Membranes—A549 cells were treated with C2-ceramide (30 μM) for various times. The mitochondrial membrane fraction was isolated as described above. Mitochondrial protein (0.5 mg) was resuspended in 400 μl of mitochondrial buffer. Cross-linkers bis(sulfosuccinimidyl) suberate and disuccinimidyl suberate were added simultaneously to a final concentration of 5 mM. Incubation was carried out at room temperature for 30 min, and then the reaction was quenched by addition of Tris-HCl, pH 8.0, to a final concentration of 20 mM as described previously (35). The heavy membrane fraction was then lysed, and lysate protein (40 μg) was subjected to SDS-PAGE followed by Western blotting with an anti-Bax antibody.

Knockdown of PP2A Catalytic Subunit by RNA Interference (RNAi)—Human PP2A/C siRNA (Santa Cruz Biotechnology) was transfected into A549 cells by using Lipofectamine™ 2000 according to the manu- facturer’s instructions. A control siRNA (non-homologous to any known gene sequence) was used as a negative control. The levels of PP2A/C expression were determined by Western blot using a PP2A/C antibody. Bax phosphorylation or cell viability was assessed following various treatments as described. Three independent experiments were conducted for specific silencing of the targeted PP2A/C gene.

Vector-based Gene Silencing of Bax by RNAi—The Bax DNA target sequence for siRNA design is AACTGATCAGAACCATCATGG. This sequence for siRNA design is AACTGATCAGAACCATCATGG. This
was determined by using the Ambion (Austin, TX) siRNA Target Finder according to the human Bax cDNA sequence. The Bax-specific hairpin siRNA insert (sense-loop-antisense) was determined using a computerized insert design tool based on a target sequence following instructions on the Ambion web site. Then the oligonucleotide encoding the Bax-specific hairpin siRNA insert was synthesized and ligated into the pSilencer™ 2.1-U6 hygro vector from Ambion. The pSilencer 2.1-U6 hygro plasmids bearing the Bax hairpin siRNA were transfected into A549 cells using Lipofectamine 2000 according to the manufacturer’s instructions. The stable clones persistently producing Bax siRNA were selected using hygromycin (0.8 mg/ml). The levels of Bax expression were analyzed by Western blot using a Bax antibody.

Cell Viability Assay—The apoptotic and viable cells were detected using an ApoAlert Annexin-V kit from Clontech according to the manufacturer’s instructions. The percentage of Annexin-V<sup>low</sup> cells (percentage of viable cells) or Annexin-V<sup>high</sup> cells (percentage of apoptotic cells) was determined by fluorescence-activated cell sorter analysis as described previously (36).

RESULTS

Treatment of Cells with Okadaic Acid or Expression of Small T Antigen Results in Bax Phosphorylation and Enhanced Cell Survival—Our previous findings and those of others have demonstrated that phosphorylation of Bax at Ser-184 through protein kinase AKT negatively regulates the proapoptotic activity of Bax (11, 12). Because Bax is extensively phosphorylated at Ser-184 through protein kinase AKT negatively regulates Bax dephosphorylation as well as its proapoptotic activity. In going Results in Bax Phosphorylation and Enhanced Cell Survival

PP2A Dephosphorylates Bax

Ceramide Inhibits Nicotine-induced Bax Phosphorylation and Enhances Apoptosis—Because ceramide is a potent pharmacological activator of PP2A (29, 31), it is possible that ceramide may induce Bax dephosphorylation through activation of PP2A. To test this, A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine in the absence or presence of C2-, C6-, C16-ceramide. Results clearly indicated that C2-ceramide suppressed nicotine-induced Bax phosphorylation in a dose-dependent manner (Fig. 2A). Importantly treatment of cells with C2-ceramide blocked nicotine-stimulated cell survival (Fig. 2B). These findings suggest that ceramide-activated PP2A may reduce Bax phosphorylation level via dephosphorylation. To further test whether other species of ceramide or TNFα (a ceramide inducer; Ref. 23) affect Bax phosphorylation, A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine in the absence or presence of C2-, C6-, C16-ceramide, or TNFα. Importantly C6-ceramide, or TNFα played a similar role in suppression of nicotine-induced Bax phosphorylation compared
with C2-ceramide (Fig. 2C). This may occur through a similar mechanism involving PP2A.

**PP2A Co-localizes and Interacts with Bax**—To assess a potential direct role for PP2A as a physiological Bax phosphatase, subcellular distribution of PP2A/C and Bax was examined by immunofluorescent staining. A mouse antibody against human Bax, rabbit polyclonal PP2A/C antibody, and FITC-conjugated anti-mouse (green) or rhodamine-conjugated anti-rabbit (red) secondary antibodies were used so that cells could be simultaneously stained without cross-reaction. As shown in Fig. 3A, Bax was primarily co-localized with PP2A/C in the cytoplasm of A549 cells. To test whether ceramide-activated PP2A enhances an association between PP2A and Bax, A549 cells were treated with C2-ceramide (30 μM) for various times. A co-immunoprecipitation experiment was carried out using an agarose-conjugated Bax antibody, respectively. Bax-associated PP2A/C (i.e. bound PP2A/C) and PP2A/C-associated Bax (i.e. bound Bax) were analyzed by Western blotting using a PP2A/C or Bax antibody, respectively. Results revealed that treatment of cells with C2-ceramide promoted PP2A/C to associate with Bax in a time-dependent manner (Fig. 3B). Thus, PP2A/C may potentially function as a physiological phosphatase for Bax to regulate its activity in human lung cancer cells. A rabbit preimmune serum was used as a control and failed to precipitate either Bax or PP2A/C (Fig. 3B).

**PP2A Directly Dephosphorylates Bax and Disrupts the Bcl2-Bax Complex**—PP2A is the most abundant known serine/threonine-specific protein phosphatase expressed in mammalian cells (38). To test whether PP2A can directly dephosphorylate Bax, A549 cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. Phosphorylated Bax was analyzed by Western using an anti-HA antibody (Fig. 4A). Overexpression of HA-PP2A/C potently disrupts the Bcl2-Bax complex in a time-dependent manner (Fig. 4A). To further test whether PP2A dephosphorylates Bax in vivo, an HA-tagged PP2A/C/pCDNA3 construct was transfected into H157 cells that express relatively low levels of endogenous PP2A (Fig. 1A). The exogenous levels of PP2A/C were analyzed by Western blot using an anti-HA antibody (Fig. 4B). H157 cells overexpressing HA-tagged PP2A/C or vector-only control cells were metabolically labeled with [32P]orthophosphoric acid and treated with nicotine for 60 min. Results indicated that overexpression of HA-PP2A/C potently blocked both nicotine-induced Bax phosphorylation and cell survival (Fig. 4C and D). To assess whether the phosphorylation status of Bax affects its heterodimerization with Bcl2, the Bcl2-Bax complex was co-immunoprecipitated from H460 cells that express high levels of endogenous Bcl2 and Bax (39) using an agarose-conjugated Bcl2 antibody and incubated with purified, active PP2A in a phosphatase assay buffer. Bcl2-associated Bax (i.e. bound Bax), free Bax, and Bcl2 were analyzed by Western blot. Intriguingly PP2A facilitated the dissociation of the Bcl2-Bax complex that is characterized by a reduced amount of bound Bax and an enhanced level of free Bax in the supernatant (Fig. 4E). These findings suggest that PP2A may directly disrupt the Bcl2-Bax complex in a mechanism involving dephosphorylation.

**Depletion of PP2A/C by RNAi Enhances Bax Phosphorylation in Association with Suppression of Apoptosis**—Our data strongly suggest that PP2A functions as a physiological Bax phosphatase to dephosphorylate Bax. To test whether PP2A is essential for Bax dephosphorylation, A549 cells were transfected with PP2A/C siRNA as described under "Experimental Procedures." Results showed that the PP2A/C siRNA efficiently and specifically reduced PP2A/C expression in A549 cells, whereas control siRNA had no effect (Fig. 5A). Importantly specific disruption of PP2A/C expression by RNAi not only resulted in Bax phosphorylation but also further enhanced nicotine-stimulated Bax phosphorylation in association with prolonged cell survival following treatment of cells with cisplatin (Fig. 5). These findings suggest that PP2A may be required for dephosphorylation of Bax to activate its proapoptotic activity.
Ceramide-induced Dephosphorylation of Bax Results in A Conformational Change of Bax Leading to Activation of the Proapoptotic Function of Bax—Our data suggest that ceramide-stimulated Bax dephosphorylation occurs in a mechanism involving activation of PP2A. To test whether dephosphorylation of Bax affects its conformational change, an anti-6A7 Bax antibody that specifically measures the conformational change of Bax leading to activation of its proapoptotic function.

Treatment of Cells with C2-Ceramide Results in Bax Translocation to Mitochondria, Insertion into Mitochondrial Membranes, Oligomerization, and Cyt c Release—The majority of Bax is located in the cytosol, and only a small portion of Bax is peripheral localized with the outer mitochondrial membranes in unstimulated cells (34). To test whether ceramide-induced dephosphorylation of Bax affects the subcellular localization of Bax, A549 cells were treated with increasing concentrations of C2-ceramide for 24 h, and immunoprecipitation of Bax was performed using a 6A7 or pan-Bax antibody, respectively. Results indicated that treatment of cells with C2-ceramide potently enhanced the ability of the 6A7 antibody to bind Bax compared with control cells (Fig. 6A). In addition, 6A7 antibody binding to Bax was further examined by immunofluorescence. Cells were treated with C2-ceramide (30 μM) for 24 h, then incubated with prewarmed (37°C) growth medium containing MitoTracker for 30 min, fixed, and permeabilized with ice-cold methanol and acetone. Fixed cells were blocked with 10% mouse serum and then immunostained with the mouse monoclonal 6A7 primary and FITC-conjugated anti-mouse secondary antibodies. Although Bax immunofluorescence was low or undetectable in untreated cells, it increased significantly in cells treated with C2-ceramide, which is associated with increased apoptotic cell death (Fig. 6B and C). These findings indicate that ceramide-induced Bax dephosphorylation may induce a conformational change in Bax leading to activation of its proapoptotic function.
PP2A Dephosphorylates Bax

![Graph showing the percentage of viable cells with C2-ceramide](image)

**FIGURE 6.** C2-ceramide induces a conformational change of Bax and promotes apoptosis. **A**. A549 cells were treated with increasing concentrations of C2-ceramide for 24 h. A co-immunoprecipitation experiment was carried out using the 6A7 or full-length Bax antibody, respectively. Bax was analyzed by Western blotting using a Bax antibody. B, A549 cells were treated with C2-ceramide (30 µM) for 24 h. Cells were incubated with prewarmed (37 °C) growth medium containing MitoTracker (red) for 30 min. Cells were then washed with 1 × PBS, fixed, permeabilized with ice-cold methanol and acetone, blocked with 10% mouse serum, and stained with mouse monoclonal 6A7 primary and FITC-conjugated anti-mouse secondary (green) antibodies. Images were merged using Openlab 3.1.5 software. Areas of co-localization are yellow. **C.** A549 cells were treated with increasing concentrations of C2-ceramide for 24 h. Cell viability was assessed as described in the legend for Fig. 1F. Data represent the mean ± S.D. of three separate determinations. IP, immunoprecipitate; Treat, treatment.

Because our data indicate that C2-ceramide can facilitate an association of PP2A/C and Bax (Fig. 3B), it is possible that ceramide-activated PP2A may interact with and dephosphorylate Bax in cytosol, and then the Bax-PP2A/C complex translocates to mitochondria. It has been demonstrated that an alkali extraction approach can distinguish whether Bax protein slightly associates with or inserts into mitochondrial membranes (34, 43). An alkali extraction experiment was used to test whether ceramide-induced Bax dephosphorylation can promote Bax insertion into mitochondrial membranes. A549 cells were treated with C2-ceramide for various times as indicated. Mitochondria were isolated, incubated in 0.1 M Na2CO3, pH 11.5, on ice for 30 min, and centrifuged at 200,000 g to yield a mitochondrial pellet as described under “Experimental Procedures.” The resulting alkali-extracted mitochondrial membrane pellet was resuspended in 1% Nonidet P-40 lysis buffer. The alkali-resistant, integral Bax was analyzed by Western blot using a Bax antibody.

![Graph showing the proportion of C2-ceramide treated cells](image)

**FIGURE 7.** Treatment of cells with C2-ceramide results in Bax translocation into mitochondria, insertion into mitochondrial membranes, and oligomerization, which promote Cyt c release. **A.** A549 cells were treated with increasing concentrations of C2-ceramide for 24 h. Mitochondrial and cytosolic fractions were isolated as described under “Experimental Procedures.” Bax was analyzed by Western blot using Bax or PP2A/C antibody, respectively. B, A549 cells were treated with C2-ceramide (30 µM) for various times. Mitochondria were isolated, and a cross-linking study using bis(sulfosuccinimidyl) suberate and disuccinimidyl suberate was carried out as described under “Experimental Procedures.” *Bax* was analyzed by Western blot with a Bax antibody. *, nonspecific band. **B.** A549 cells were treated with C2-ceramide (30 µM) for various times. Mitochondrial and cytosolic fractions were isolated as described under “Experimental Procedures.” Levels of Cyt c (Cyto c) in these two fractions were analyzed by Western blot using a Cyt c antibody.

Cells with C2-ceramide facilitated Bax molecules to form dimers and trimers as well as multimers. The molecular sizes of these adducts obtained were estimated to be multiples of ~21 kDa (Fig. 7C), suggesting the formation of Bax homo-oligomers. These findings reveal that the dephosphorylated form of Bax may more efficiently undergo oligomerization in the mitochondrial membranes. Functionally, C2-ceramide-induced Bax oligomerization in the mitochondrial membranes enhanced Cyt c release (Fig. 7D). These findings uncover a novel mechanism by which ceramide-induced dephosphorylation of Bax increases its proapoptotic potency.

**PP2A Facilitates Bax Insertion into Mitochondrial Membranes and Cyt c Release**—Bax is not only located in the cytosol but is also peripherally associated with the outer mitochondrial membranes in unstimulated cells (34). C2-ceramide could induce the non-integral Bax molecules from the cytosol and/or peripheral location to the mitochondrion for insertion into the outer mitochondrial membranes, which induces mitochondrial dysfunction (Fig. 7). To assess whether PP2A can render Bax insertion into mitochondrial membranes to induce Cyt c release by
PP2A Dephosphorylates Bax

**DISCUSSION**

Bax, the major promoter of cell death, has been identified as a promising prognostic indicator in patients with lung cancer (44). However, the mechanism(s) involved in regulating the proapoptotic activity of Bax is not fully understood. It has been proposed that activation of the proapoptotic function of Bax likely occurs through several interdependent mechanisms including translocation from cytosol to mitochondria (34), oligomerization, and insertion into mitochondrial membranes following various stresses (45-47). In healthy cells, Bax is predominantly a soluble monomeric protein despite the fact that it possesses a C-terminal hydrophobic segment (40). This hydrophobic domain, unlike those of Bcl2 and Bcl-XL, is sequestered inside a hydrophobic cleft (3, 45). Bax is translocated into mitochondria upon induction of apoptosis (48, 49). This translocation process appears to involve a conformational change in Bax to expose its C-terminal hydrophobic domain (50). After translocation, Bax forms large oligomers that insert into mitochondrial membranes leading to Cyt c release and cytotoxic activities. However, this has not been seen in other Bcl2 family proteins such as Bid and Bad (46).

Because our previous studies as well as those of others discovered that dephosphorylating peripherally associated Bax, intact mitochondria were isolated from A549 cells and incubated with purified active PP2A in a phosphatase assay buffer for various times. After treatment of mitochondria with PP2A, an alkali extraction of the Cyt c release assay was carried out as described above. Results revealed that PP2A not only rendered Bax resistant to alkali extraction but also induced Cyt c release from isolated mitochondria (Fig. 8). These findings suggest that PP2A-induced Cyt c release may occur by a mechanism involving dephosphorylation of Bax.

In the present study, stable gene silencing approach was used to specifically deplete Bax from A549 cells. The pSilencer 2.1-U6 hygro plasmid containing a Bax hairpin siRNA insert or control hairpin siRNA insert was transfected into A549 cells using Lipofectamine 2000. The levels of Bax expression were analyzed by Western blot using a Bax antibody. A549 cells expressing Bax siRNA or control siRNA were treated with increasing concentrations of C2- or C12- ceramide for 24 h. Cell viability was assessed as described in the legend for Fig. 1F. Data represent the mean ± S.D. of three separate determinations.

Depletion of Bax by RNAi resulted in resistance to apoptosis induced by C2-ceramide (Fig. 9A). The pSilencer 2.1-U6 hygro plasmids bearing the Bax hairpin siRNA insert or control hairpin siRNA insert were transfected into A549 cells using Lipofectamine 2000. The levels of Bax expression were analyzed by Western blot using a Bax antibody. A549 cells expressing Bax siRNA or control siRNA were treated with increasing concentrations of C2-ceramide for 24 h. Cell viability was assessed as described in the legend for Fig. 1F. Data represent the mean ± S.D. of three separate determinations.

**PP2A Dephosphorylates Bax**

**FIGURE 8.** PP2A promotes Bax insertion into mitochondrial membranes and Cyt c release. A, the intact mitochondria were isolated from A549 cells and incubated with purified PP2A at 30 °C for various times. Alkali extraction of Bax was performed as described under “Experimental Procedures.” The alkali-resistant Bax (i.e. nonextractable) was determined by Western blot using a Bax antibody. B, the intact mitochondria were isolated from A549 cells and incubated with purified PP2A at 30 °C for various times. Levels of Cyt c (Cyt c) in mitochondria and supernatant were analyzed by Western blotting using a Cyt c antibody.

**FIGURE 9.** Depletion of Bax by RNAi results in resistance to apoptosis induced by C2-ceramide. A, the pSilencer 2.1-U6 hygro plasmids bearing the Bax hairpin siRNA insert or control hairpin siRNA insert were transfected into A549 cells using Lipofectamine 2000. The levels of Bax expression were analyzed by Western blot using a Bax antibody. B, A549 cells expressing Bax siRNA or control siRNA were treated with increasing concentrations of C2-ceramide for 24 h. Cell viability was assessed as described in the legend for Fig. 1F. Data represent the mean ± S.D. of three separate determinations.
PP2A Dephosphorylates Bax

FIGURE 10. Proposed model of PP2A-induced Bax dephosphorylation in regulating apoptosis. PP2A-induced Bax dephosphorylation causes a conformational change in Bax and facilitates Bax translocation to mitochondria, insertion into mitochondrial membranes, and formation of oligomers, which lead to Cyt c release and apoptosis. Furthermore, PP2A-induced Bax dephosphorylation disrupts Bcl2/Bax binding. This may prevent Bcl2 from suppressing the proapoptotic function of Bax.

potently induced Bax dephosphorylation (Figs. 2A and 3B), suggesting that Bax may function as a downstream death target in the ceramide-induced apoptosis signaling pathway. Importantly, ceramide-induced Bax dephosphorylation caused a conformational change detected by the 6A7 Bax antibody, which only recognizes active, conformationally changed Bax (Fig. 6), suggesting that dephosphorylation of Bax may result in exposure of the 6A7 epitope (amino acids 13–19), which is normally hidden in its N terminus. Immunofluorescence analysis using the 6A7 Bax antibody following treatment of cells with C2-ceramide exhibited punctate staining that was localized within mitochondria in association with increased cell death (Fig. 6, B and C), indicating that the 6A7 epitope-specific conformational change resulting from Bax dephosphorylation enables it to bind the mitochondria and promotes apoptosis. In support of this, subcellular fractionation studies revealed that C2-ceramide-induced Bax accumulation on mitochondrial membranes (Fig. 7A). Although the Bax molecule has mitochondrial targeting signals in its sequence, it is not clear what factors keep it in the cytosol in normal cells. We previously demonstrated that AKT-induced Bax phosphorylation at Ser-184 retains Bax in cytosol as an inactive form (11). Direct evidence obtained from metabolic labeling experiments indicated that the phosphorylated form of Bax was exclusively localized in cytosol (Fig. 1C). Ceramide not only enhanced PP2A/Bax binding but also induced Bax dephosphorylation and translocation to mitochondria (Figs. 2, 3, and 7), supporting the notion that ceramide-activated PP2A may initially associate with and dephosphorylate the phosphorylated form of Bax in cytosol and then the PP2A-Bax complex is translocated to mitochondria (Fig. 10). Moreover, ceramide-induced Bax dephosphorylation may activate Bax via a conformational change, which promotes Bax to associate with and insert into mitochondrial membranes (Figs. 6 and 7). Using chemical cross-linkers, we found that treatment of cells with ceramide facilitated the formation of various Bax oligomers (i.e. from dimers to pentamers; Fig. 7C). It has been demonstrated that Bax oligomers can form a pore size that is capable of transporting Cyt c (54). In support of this, our findings indicate that ceramide-stimulated Bax oligomerization is associated with increased Cyt c release (Fig. 7, C and D). It is possible that the dephosphorylated form of Bax induced by ceramide may more efficiently form large structures (i.e. oligomers) in mitochondrial membranes to allow the passage of proteins at least of the size of Cyt c. These results reveal a novel mechanism by which Bax dephosphorylation enhances its proapoptotic activity.

Bax is found in both the cytosolic and mitochondrial fractions in many culture cells (34, 40, 55). Alkali extraction studies have further demonstrated that most Bax is lost from mitochondrial membranes during this treatment, indicating that under normal conditions Bax is loosely attached to mitochondria (34, 43). To directly test whether PP2A-induced dephosphorylation promotes the "peripherally" associated mitochondrial Bax to insert into mitochondrial membrane, isolated mitochondria from unstimulated A549 cells were treated with purified PP2A for various times. Results showed that addition of PP2A rendered Bax resistant to alkali extraction, indicating that it undergoes membrane integration (Fig. 8A). Correlatively, PP2A-induced Bax membrane integration facilitated Cyt c release (Fig. 8B). Thus, in addition to dephosphorylation of cytosolic phosphorylated Bax, activated PP2A may also dephosphorylate the peripherally associated mitochondrial Bax and promote Bax to insert into mitochondrial membranes and induce Cyt c release. Cells expressing Bax siRNA were highly resistant to apoptosis induced by the PP2A activator C2-ceramide (Fig. 9), indicating that Bax is a required downstream target in the ceramide/PP2A-mediated death signaling.

In summary, our findings identified PP2A as a novel physiological Bax phosphatase that can directly dephosphorylate and activate the major proapoptotic molecule, Bax. Ceramide-induced Bax dephosphorylation through activation of PP2A resulted in a conformational change by exposure of the 6A7 epitope (amino acids 13–19) in Bax that facilitated its translocation to mitochondria, insertion into mitochondrial membranes, and formation of oligomers leading to Cyt c release and apoptotic cell death (Fig. 10). Thus, therapeutic activation of PP2A to dephosphorylate Bax by enhancing ceramide production may have clinical relevance for the treatment of human lung cancer and other Bax-expressing malignancies.

Acknowledgments—We are grateful to Drs. Brian Law (University of Florida) and Marc Mumby (University of Texas Southwest Medical Center) for kindly providing the HA-tagged PP2A/C/pCDNA3 and small t antigen/pCMV5 constructs.

REFERENCES
1. Adams, J. M., and Cory, S. (1998) Science 281, 1322–1326
2. Gross, A., McDonnell, J. M., and Kosmeryer, J. (1999) Genes Dev. 13, 1899–1911
3. Suzuki, M., Youle, R. J., and Tjandra, (2000) Cell 103, 645–654
4. Farrow, S. N., and Brown, R. (1996) Curr. Opin. Genet. Dev. 6, 45–49
5. Oliva, Z., Nollman, C. L., and Kosmeryer, S. J. (1995) Cell 74, 609–619
6. Kroemer, G. (1997) Nat. Med. 3, 614–620
7. Li, H., Zhu, H., Xu, C., and Yuan, J. (1998) Cell 94, 491–501
8. Zong, W. X., Lindstein, T., Ross, A. J., MacGregor, G. R., and Thompson, C. B. (2001) Genes Dev. 15, 1481–1486
9. Wei, M., Zong, W. X., Cheng, E. H., Lindsten, T., Panoutsakopoulou, V., Ross, A. J., Roth, K. A., MacGregor, G. R., Thompson, C. B., and Kosmeryer, S. J. (2001) Science 292, 727–730
10. Xi, M., and Deng, X. (2005) J. Biol. Chem. 280, 10781–10789
11. Gardai, S. J., Hildeman, D. A., Franklin, S. K., Whitlock, B. B., Frasch, S. C., Borregaard, N., Wang, K., Gross, A., Waksman, G., and Kosmeryer, S. J. (1998) Mol. Cell. Biol. 18, 6083–6089
12. Linseman, D., Butts, B., Precht, T., Phelps, R., Le, S., Laessig, T., Bouchard, R., McClure, M., and Heidenreich, K. (2004) J. Neurosci. 24, 9993–10002
13. Mumby, M. C., and Walter, G. (1993) Physiol. Rev. 73, 673–699
15. Cegielska, A., Shaffer, S., Derua, R., Goris, J., and Virshup, D. (1994) Mol. Cell. Biol. 14, 4616–4623
16. Sontag, E., Nunbhakdi-Craig, V., Bloom, G., and Mumbry, M. (1995) J. Cell Biol. 128, 1131–1144
17. McCright, B., Rivers, A., Audlin, S., and Virshup, D. (1996) J. Biol. Chem. 271, 22081–22089
18. Mayer-Jaekel, R., Baumgartner, S., Bilbe, G., Ohkura, H., Glover, D., and Hemmings, B. (1992) Mol. Biol. Cell 3, 287–298
19. Hannun, Y. A. (1994) J. Biol. Chem. 269, 3125–3128
20. Kolesnick, R., and Fuks, Z. (1995) J. Exp. Med. 181, 1949–1952
21. Jarvis, W. D., Grant, S., and Kolesnick, R. N. (1996) Clin. Cancer Res. 2, 1–6
22. Smyth, M. L., Obeid, L. M., and Hannun, Y. A. (1997) Adv. Pharmacol. 41, 133–154
23. Obeid, L. M., Lindardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Science 259, 1769–1771
24. Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M., and Hannun, Y. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 34157–34163
25. Ikonen, E., Fiedler, K., Parton, R. G., and Simons, K. (1995) FEBS Lett. 358, 273–277
26. Ruvolo, P., Clark, W., Mumbry, M., Gao, F., and May, W. S. (2002) J. Biol. Chem. 277, 22847–22852
27. Law, B., and Rossie, S. (1995) J. Biol. Chem. 270, 12808–12813
28. Deng, X., Ito, T., Carr, B., Mumbry, M., and May, W. S. (1998) J. Biol. Chem. 273, 34157–34163
29. Ilonen, E., Feidler, K., Parton, R. G., and Simons, K. (1995) FEBS Lett. 358, 273–277
30. Goping, I. S., Gross, A., Lavoie, J. N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S. J., and Shore, G. C. (1998) J. Cell Biol. 143, 207–215
31. Cao, X., Deng, X., and May, W. S. (2003) Blood 102, 2605–2614
32. Deng, X., Xiao, L., Lang, W., Gao, F., Ruvolo, P., and May, W. S. (2001) J. Biol. Chem. 276, 23681–23688
33. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumbry, M. (1993) Cell 75, 887–897
34. Kremmer, E., Ohst, K., Kiefer, J., Brenis, N., and Walter, G. (1997) Mol. Cell. Biol. 17, 1692–1701
35. Jin, Z., Xin, M., and Deng, X. (2005) J. Biol. Chem. 280, 16045–16052
36. Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 3668–3672
37. Hsu, Y. T., and Youle, R. J. (1998) J. Biol. Chem. 273, 10777–10783
38. Sawada, M., Sun, W., Hayes, P., Leskov, K., Boothman, D., and Matsuyama, S. (2003) Nat. Cell Biol. 5, 320–329
39. Ekes, R., Desaghet, S., Antonsson, B., and Martinou, J. (2000) Mol. Cell. Biol. 20, 929–935
40. Groeger, A. M., Esposito, V., Cassandro, R., Baldi, G., Rosselli, L., De Luca, L., Kadletz, M., and Kaiser, H. E. (2001) Anticancer Res. 21, 3627–3630
41. Miao, S. S., Hsu, Y. T., Carvalho, A. C. L., Rosenstock, T. R., Sharpe, J. C., and Youle, R. J. (2003) Braz. J. Med. Biol. Res. 36, 183–190
42. Nischukasen, K., Smith, C. L., Lamensdorf, L., Yan, X. H., and Youle, R. J. (2001) J. Cell Biol. 153, 1265–1276
43. Mikhailov, V., Mikhailova, M., Pulkrabek, D. J., Dong, Z., Venkataraman, M. A., and Salkin, P. (2001) J. Biol. Chem. 276, 18361–18374
44. Eldering, E., Mackus, W. J., Derks, I. A., Beuling, E., Teeling, P., Lens, S. M., Van Oers, M. H., and Van Lier, R. A. (2004) Eur. J. Immunol. 34, 1950–1960
45. Deng, X., and Wu, X. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12050–12055
46. Nischukasen, K., Smith, C. L., Hsu, Y. T., and Youle, R. J. (1999) EMBO J. 18, 2330–2341
47. Gulbins, E. (2003) Pharmacol. Res. 47, 393–399
48. Ruvolo, P. (2003) Pharmacol. Res. 47, 383–392
49. Kolesnick, R., and Kronke, M. (1998) Annu. Rev. Physiol. 60, 643–645
50. Saito, M., Korsmeyer, S. J., and Schlesinger, P. H. (2000) Nat. Cell Biol. 2, 553–555
51. Wolter, K., Hsu, Y., Smith, C., Nischukasen, A., Xi, X., and Youle, R. J. (1997) J. Cell Biol. 139, 1281–1292