Protein phosphatase 2A inactivates Bcl2's antiapoptotic function by dephosphorylation and up-regulation of Bcl2-p53 binding

Xingming Deng,[1] Fengqin Gao,[1] and W. Stratford May[1]

[1]Department of Medicine, Division of Hematology/Oncology, University of Florida Shands Cancer Center, Gainesville

Bcl2 is associated with chemoresistance and poor prognosis in patients with various hematologic malignancies. DNA damage-induced p53/Bcl2 interaction at the outer mitochondrial membrane results in a Bcl2 conformational change with loss of its antiapoptotic activity in interleukin-3–dependent myeloid H7 cells. Here we find that specific disruption of protein phosphatase 2A (PP2A) activity by either expression of small t antigen or depletion of PP2A/C by RNA interference enhances Bcl2 phosphorylation and suppresses cisplatin-stimulated p53/Bcl2 binding in association with prolonged cell survival. By contrast, treatment of cells with C2-ceramide (a potent PP2A activator) or expression of the PP2A catalytic subunit (PP2A/C) inhibits Bcl2 phosphorylation, leading to increased p53/Bcl2 binding and apoptotic cell death. Mechanistically, PP2A-mediated dephosphorylation of Bcl2 in vitro promotes its direct interaction with p53 as well as a conformational change in Bcl2. PP2A directly interacts with the BH4 domain of Bcl2 as a docking site to potentially "bridge" PP2A to Bcl2's flexible loop domain containing the target serine 70 phosphorylation site. Thus, PP2A may provide a dual inhibitory effect on Bcl2's survival function by both dephosphorylating Bcl2 and enhancing p53-Bcl2 binding. Activating PP2A to dephosphorylate Bcl2 and/or increase Bcl2/p53 binding may represent an efficient and novel approach for treatment of hematologic malignancies. (Blood. 2009;113:422-428)

Introduction

Bcl2 was initially discovered in the t(14;18) fusion oncogene expressed in follicular lymphomas and was subsequently discovered to be responsible for prolonged cell survival and drug resistance in interleukin-3 (IL-3)–dependent hemopoietic cells expressing this founding antiapoptotic family member.[1-4] Bcl2 is up-regulated in many/most hematologic malignancies by posttranslational modification, including phosphorylation and by protein-protein interaction with proapoptotic Bcl2 members, such as Bax or Bak.[4-6] Activation of either the extrinsic (eg, death receptor: mediated by tumor necrosis factor-α or Fas-L) or the intrinsic (ie, mitochondrial controlled) death pathway (ie, by growth factor withdrawal, chemotherapy, irradiation, or viral infection) can lead to mitochondrial dysfunction with activation of apoptosis.[7,8] Bcl2 can suppress cell death induced by a variety of stress applications. However, it is not yet clear how Bcl2 is regulated to functionally block apoptosis and promote survival. One mechanism by which growth factor (ie, IL-3, erythropoietin, nerve growth factor, or serum) signaling can regulate Bcl2 members is by phosphorylation, which positively regulates Bcl2 and negatively regulates the proapoptotic proteins, Bax and Bad.[3,9,10] For Bcl2, the "regulatory" flexible loop domain (FLD) where monosite or multisite phosphorylation occurs lies between the N-terminal BH4 and BH3 regions.[7,11] Monosite phosphorylation of Bcl2 at S70 can be mediated by several growth factor-activated protein kinases, including the mitogen-activated protein kinases, ERK 1/2, protein kinase C-α, or the stress-activated JNK1 Bcl2 kinase.[12-14] Furthermore, multisite phosphorylation of Bcl2 in the FLD can occur at 3 sites, S70, T69, and S87 when cells are treated with a microtubule disrupting agent, such as paclitaxel.[15] By performing genetic studies with compound phosphomimetic Bcl2 mutants, we discovered that phosphorylation at any of these 3 sites can significantly enhance Bcl2's antiapoptotic function but that only S70 is phosphorylated in the presence of growth factors (ie, the physiologic phosphorylation site).[3,11]

Protein phosphatase 2A (PP2A) is a major protein serine/threonine phosphatase that participates in many mammalian signaling pathways.[16] PP2A 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 kDa to 130 kDa). The AC catalytic complex alone contains the phosphatase activity, whereas the distinct regulatory B-subunit can recruit PP2A/C to a selective subcellular location that defines a specific substrate target.[17-19] The A and C subunits are evolutionarily conserved and ubiquitously expressed.[20] These 2 subunits form a catalytic complex (PP2A/AC) that can interact with at least 3 families of regulatory subunits (B, B′, and B″) or certain tumor antigens (ie, SV40 small tumor antigen) to affect activity and determine PP2A substrate specificity.[16,17] We previously demonstrated that Bcl2 phosphorylation is a dynamic process that involves not only a Bcl2 kinase but also a physiologic Bcl2 phosphatase (PP2A) that can dephosphorylate Bcl2.[21] Furthermore, the potent tumor suppressor p53 has recently been shown to have an "extranuclear" function to bind to and negatively affect Bcl2's survival function in a mechanism regulated by Bcl2's phosphorylation status.[22] However, the mechanism of PP2A-mediated Bcl2 dephosphorylation and how it regulates Bcl2's antiapoptotic function are not yet clear. Here we demonstrate that PP2A inactivates Bcl2 by direct dephosphorylation as a result of binding to Bcl2's BH4 domain to access its target phosphorylation site(s) in the FLD. This mechanism also

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facilitates p53/Bcl2 binding. These findings suggest that targeting PP2A may represent a novel therapeutic approach in the majority of hematologic malignancies that express Bcl2.

Methods

Antibodies and other reagents

Okadaic acid, C2-ceramide, and purified PP2A were obtained from Calbiochem (San Diego, CA). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was obtained from Sigma-Aldrich (St Louis, MO). PP2A/C small interfering RNA (siRNA), anti-Bcl2, anti-p53, fluorescein isothiocyanate (FITC)–conjugated antirabbit antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-small t antigen antibody was purchased from Research Diagnostics (Flanders, NJ). The Bcl2/BH3-domain specific antibody was obtained from Abgent (San Diego, CA). Purified recombinant wild-type (WT) Bcl2 protein was obtained from Protein X Laboratory (San Diego, CA). Synthetic murine IL-3 was kindly provided by Ian Clark-Lewis (University of British Columbia, Vancouver, BC). The hemagglutinin (HA)–tagged PP2A/C–pCDNA3 construct was generously provided by Dr Brian Law (University of Florida, Gainesville). WT small t antigen cDNA in pCMV5 was kindly provided by Dr Marc Mumby (University of Texas Southwestern Medical Center, Dallas). All reagents used were obtained from commercial sources unless otherwise stated.

Plasmids, cDNA, cell line, and stable transfections

The pCIneo plasmid containing WT Bcl2 cDNA was transfected into IL-3–dependent murine myeloid NSF.H7 cells by electroporation. Clones stably expressing WT Bcl2 were selected in medium containing G418 (0.6 mg/mL).

Metabolic labeling, immunoprecipitation, and Western blotting

Cells were washed with phosphate-free RPMI medium and metabolically labeled with [^{32}P] orthophosphoric acid for 60 minutes. After treatment of cells with IL-3, cells were washed with ice-cold 1× phosphate-buffered saline (PBS) and lysed in detergent buffer. Bcl2 was immunoprecipitated using an agarose-conjugated Bcl2 antibody. The samples were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and exposed to Kodak X-Omat film at −80°C. Bcl2 phosphorylation was determined by autoradiography. The same filter was then probed by Western blot using a Bcl2 antibody and developed using an enhanced chemiluminescence kit from GE Healthcare (Little Chalfont, United Kingdom) as described previously.[9]

Dephosphorylation of Bcl2 in vitro

H7 cells expressing WT Bcl2 were metabolically labeled with [^{32}P] orthophosphoric acid for 90 minutes and treated with IL-3 for 15 minutes. ^{32}P-labeled Bcl2 was immunoprecipitated using an agarose-conjugated Bcl2 antibody. The beads were washed 3 times in detergent buffer and resuspended in 60 μL phosphatase assay buffer containing 50 mM Tris-HCl, pH 7.0, 20 mM β-mercaptoethanol, 2 mM $MnCl_2$, 0.1% bovine serum albumin. Purified PP2A (50 ng) was added, and the samples were incubated at 30°C for various times as described.[21] The reaction was terminated by the addition of 2× SDS sample buffer. The sample was boiled for 5 minutes before loading onto SDS-PAGE. Bcl2 phosphorylation was determined by autoradiography.

Immunofluorescence

H7 cells expressing WT Bcl2 were treated with increasing concentrations of C2-ceramide. Cells were then washed with 1× PBS, fixed and permeabilized with ice-cold methanol and acetone, and blocked with 10% rabbit serum. Cells were stained with 4,6-diamino-2-phenylindole, the Bcl2/BH3 primary antibody, and FITC-conjugated secondary antibody. Cells were washed with 1× PBS and observed under a fluorescent microscope (Carl Zeiss, Thornwood, NY). Pictures were taken and colored with the same exposure setting for each experiment.

Knockdown of PP2A catalytic subunit by RNA interference

PP2A/C siRNA (Santa Cruz Biotechnology) was transfected into WT Bcl2-expressing H7 cells using Lipofectamine-2000 according to the manufacturer's instructions. A control siRNA (nonhomologous to any known gene sequence) was used as a negative control. The level of PP2A/C expression was determined by Western blot using a PP2A/C antibody. Bcl2 phosphorylation or cell viability was assessed after various treatments as described. Three independent experiments were conducted to determine specific silencing of the targeted PP2A/C gene.

Cell viability assay

The apoptotic and viable cells were detected using an ApoAlert Annexin-V kit from Clontech (Mountain View, CA) according to the manufacturer's instructions. The percentage of annexin V^{low} cells (percentage of viable cells) or annexin V^{high} cells (percentage of apoptotic cells) was determined by fluorescence-activated cell sorter analysis (FACS) as described.[14] Cell viability was also confirmed using trypan blue dye exclusion and TdT-mediated dUTP nick end labeling assays.

Results

Disruption of PP2A activity by expression of small t antigen results in increased Bcl2 phosphorylation, decreased Bcl2/p53 binding, and prolonged survival of IL-3–dependent myeloid cells

Our previous findings demonstrate that treatment of cells with okadaic acid (OA; ie, an inhibitor for both PP2A and PP1) results in increased Bcl2 phosphorylation and that PP2A could directly dephosphorylate Bcl2 in vitro.[21] These findings suggest that PP2A may function as a physiologic Bcl2 phosphatase to regulate Bcl2's antiapoptotic activity. It is well-known that the SV40 small tumor antigen (small t) can directly interact with the 36-kDa catalytic C and the 63-kDa A subunits of PP2A to specifically disrupt PP2A but not PP1 activity.[23] To more specifically test whether PP2A is a physiologic Bcl2 phosphatase, the pCMV5/small t antigen construct was transfected into H7 cells expressing WT Bcl2. After transfection, cells were metabolically labeled with [^{32}P] orthophosphoric acid for 90 minutes. Intriguingly, expression of small t antigen results in increased Bcl2 phosphorylation, suppression of cisplatin-induced Bcl2/p53 binding, increased Bcl2/Bax interaction, and prolongation of cell survival (Figure 1). These findings not only support the notion that PP2A functions as a physiologic Bcl2 phosphatase but also that PP2A activity can enhance Bcl2's interaction with p53 after DNA damage in association with dephosphorylation. In untreated cells, only low or undetectable levels of Bcl2 phosphorylation observed may result from PP2A activity because Bcl2 phosphorylation is a dynamic process dependent on both a Bcl2 kinase (ie, protein kinase C or ERK1/2)[12,13] and a phosphatase (ie, PP2A).[21] Thus, protein kinase-mediated constitutive phosphorylation of Bcl2 may occur in untreated cells because specific inhibition of PP2A activity by small t antigen up-regulates Bcl2 phosphorylation (Figure 1A,B). Similar experiments were also performed using other cell lines (ie, HL60 or REH cells), and similar results were obtained (data not shown).

PP2A directly dephosphorylates Bcl2 in vitro and in vivo to enhance Bcl2/p53 binding and apoptotic cell death

PP2A is the most abundant serine/threonine protein phosphatase expressed in mammalian cells.[16] Here we confirm our previous finding that purified, active PP2A can directly remove the ^{32}P-labeled phosphate from Bcl2 in vitro (Figure 2A).[21] To test whether

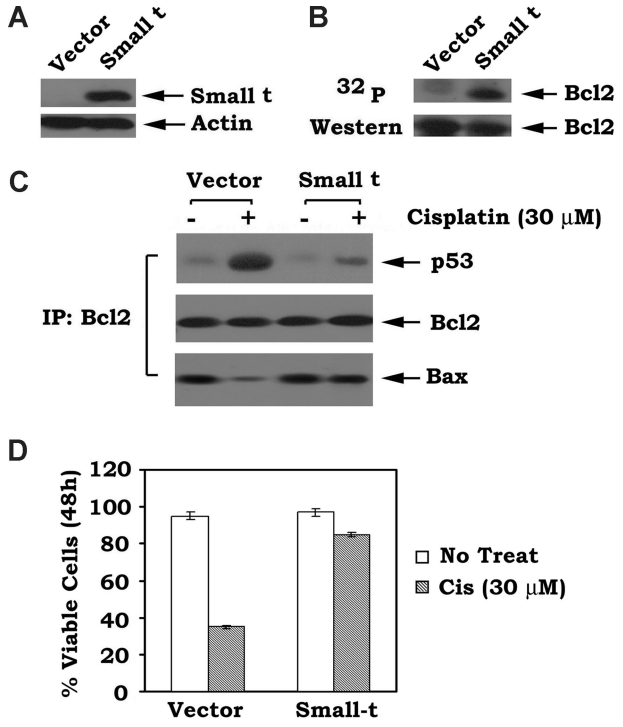

Figure 1. Expression of small t enhances Bcl2 phosphorylation and suppresses Bcl2/p53 binding in association with prolonged cell survival. (A) The pCMV5/small t construct or vector-only control was transfected into H7 cells expressing WT Bcl2 using Lipofectamine 2000. Expression levels of small t antigen were analyzed by Western blot using a small t antibody. (B) H7 WT Bcl2 cells expressing small t antigen or vector-only control were metabolically labeled with ^{32}P-orthophosphoric acid for 120 minutes. Bcl2 was immunoprecipitated using an agarose-conjugated Bcl2 antibody. Phosphorylation of Bcl2 was determined by autoradiography (upper). Western blot analysis was performed to confirm and quantify Bcl2 protein (lower). (C) H7 WT Bcl2 cells expressing small t antigen or vector-only control cells were treated with cisplatin (30 μM) for 24 hours followed by lysis in 1% CHAPS-containing buffer. Coimmunoprecipitation was performed using an agarose-conjugated Bcl2 antibody. p53, Bcl2, and Bax were then analyzed by Western blotting. (D) H7 WT Bcl2 cells expressing small t antigen or vector control were treated with cisplatin (30 μM) for 48 hours. Cell viability was determined by analyzing annexin V binding on FACS. Data represent the mean plus or minus SD of 3 separate determinations.

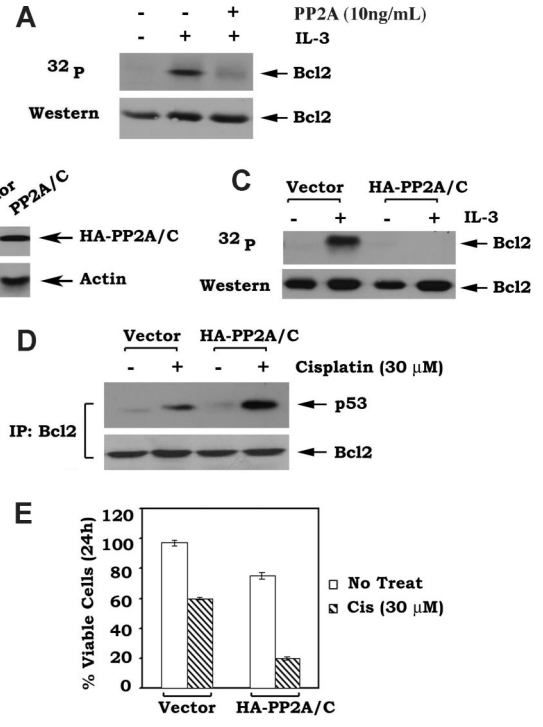

Figure 2. Overexpression of HA-tagged PP2A/C results in dephosphorylation of Bcl2, increased p53/Bcl2 binding, and apoptotic cell death. (A) H7 WT Bcl2 cells were metabolically labeled with ^{32}P-orthophosphoric acid and treated with IL-3 for 15 minutes. Phosphorylated Bcl2 was immunoprecipitated and incubated with purified PP2A (10 ng) at 30°C for 10 minutes. Phosphorylation of Bcl2 was determined by autoradiography. (B) The HA-tagged PP2A/C–pCDNA3 construct or vector control was transfected into H7 WT Bcl2 cells using Lipofectamine 2000. Expression levels of exogenous HA-PP2A/C were analyzed by Western blot using an anti-HA antibody. (C) H7 WT Bcl2 cells overexpressing HA-PP2A/C or vector control were metabolically labeled with ^{32}P-orthophosphoric acid and treated with IL-3 for 15 minutes. Phosphorylation of Bcl2 was analyzed by autoradiography. (D) H7 WT Bcl2 cells overexpressing HA-PP2A/C or vector control were treated with cisplatin (30 μM) for 24 hours followed by lysis in 1% CHAPS-containing buffer. Coimmunoprecipitation was performed using an agarose-conjugated Bcl2 antibody. The Bcl2-associated p53 and Bcl2 were analyzed by Western blotting. (E) H7 WT Bcl2 cells overexpressing HA-PP2A/C or vector control were treated with cisplatin (30 μM) for 24 hours. Cell viability was determined by analyzing annexin V binding on FACS. Data represent the mean plus or minus SD of 3 separate determinations.

PP2A can dephosphorylate Bcl2 in vivo, an HA-tagged PP2A/C–pCDNA3 construct was transfected into H7 cells expressing Bcl2. The exogenous level of PP2A/C expressed was analyzed by Western blot using an anti-HA antibody (Figure 2B). Bcl2-H7 cells expressing HA-tagged PP2A/C or vector-only control cells were metabolically labeled with [^{32}P] orthophosphoric acid and treated with IL-3 for 15 minutes. Results indicate that expression of HA-PP2A/C blocks IL-3–induced phosphorylation of Bcl2 (Figure 2C). Intriguingly, expression of HA-tagged PP2A/C also potently enhances cisplatin-stimulated Bcl2/p53 binding and apoptotic cell death (Figure 2D,E), indicating a functional role for PP2A in Bcl2 dephosphorylation and p53 interaction.

Purified PP2A promotes formation of the Bcl2/p53 complex in vitro in an OA-sensitive manner and activation of PP2A by ceramide enhances Bcl2/p53 binding

To directly test whether PP2A can affect the Bcl2/p53 interaction in vitro, purified recombinant Bcl2 and p53 were incubated in the absence or presence of increasing concentrations of purified, active PP2A. Coimmunoprecipitation reveals that the addition of PP2A can specifically promote Bcl2/p53 interaction because the PP2A inhibitor OA potently suppresses any PP2A-enhanced Bcl2/p53 binding (Figure 3A). These data suggest that PP2A activity is essential for its facilitating effect on Bcl2/p53 binding. Next, to assess whether pharmacologic regulation of PP2A activity can also affect Bcl2/p53 interaction in vivo, H7 cells expressing WT Bcl2 were treated with cisplatin in the absence or presence of either OA (ie, a PP2A inhibitor) or C2-ceramide (ie, a PP2A activator) for 24 hours. Results indicate that treatment of cells with OA inhibits cisplatin-induced Bcl2/p53 binding (Figure 3B). By contrast, treatment of cells with C2-ceramide increases cisplatin-stimulated Bcl2/p53 interaction (Figure 3B). Thus, OA and C2-ceramide have opposing roles in regulating Bcl2/p53 association, which may result from their opposing effects on PP2A activity and Bcl2 phosphorylation.

PP2A binds to Bcl2's BH4 domain

Bcl2 family members share homology in the Bcl2 homology (BH) domains (ie, BH1, BH2, BH3, and BH4).[24] To assess whether PP2A can directly bind to Bcl2 at a BH domain(s), purified PP2A/C protein (10 ng) was incubated with purified recombinant WT Bcl2 or deletion mutant Bcl2 lacking a BH domain, including ΔBH1, ΔBH2, ΔBH3, or ΔBH4 Bcl2 in 1% CHAPS lysis buffer at 4°C for

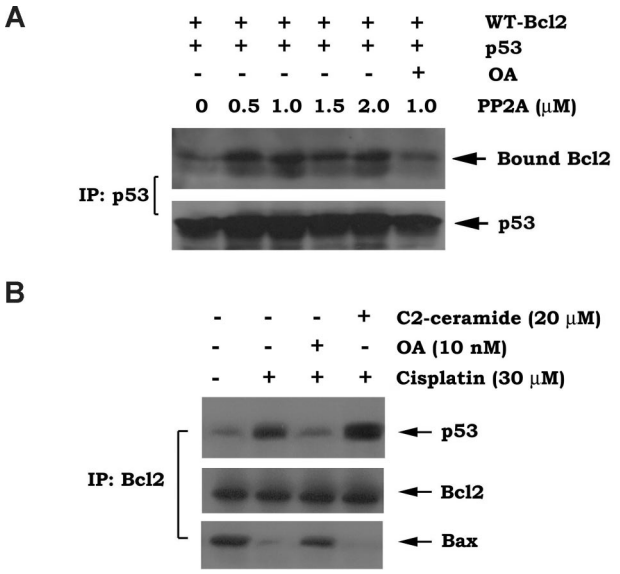

Figure 3. Purified PP2A promotes p53/Bcl2 interaction in vitro. (A) Purified p53 was incubated with full-length WT Bcl2 in the absence or presence of increasing concentrations of purified, active PP2A or OA, and coimmunoprecipitation was carried out using an agarose-conjugated p53 antibody. The p53-associated Bcl2 (bound Bcl2) and p53 were analyzed by Western blot using a Bcl2 or p53 antibody, respectively. (B) H7 WT Bcl2 cells were treated with cisplatin in the absence or presence of C2-ceramide or OA followed by lysis in 1% CHAPS-containing buffer. Coimmunoprecipitation was performed using an agarose-conjugated Bcl2 antibody. p53, Bcl2, and Bax were then analyzed by Western blotting using a p53, Bcl2, or Bax antibody as indicated.

2 hours. PP2A/C-associated Bcl2 could be coimmunoprecipitated using a PP2A/C antibody. Because WT Bcl2 could not be coimmunoprecipitated by the PP2A/C antibody in the absence of PP2A/C (Figure 4B bottom panel, lane 1 vs lane 2), these data indicate that the binding of Bcl2 to PP2A/C is specific, at least in this assay. Results demonstrate that PP2A/C associates with WT, ΔBH1, ΔBH2, or ΔBH3 Bcl2 mutants but not with the ΔBH4 Bcl2 mutant (Figure 4B bottom panel), indicating that the BH4 domain is essential for PP2A interaction.

Depletion of PP2A/C by RNAi enhances Bcl2 phosphorylation in association with suppression of cisplatin-induced Bcl2/p53 binding and increased cell survival

Our data strongly suggest that PP2A can function as a physiologic Bcl2 phosphatase to dephosphorylate Bcl2 and regulate its association with p53. To test whether PP2A is required for Bcl2 dephosphorylation, Bcl2-expressing H7 cells were transfected with PP2A/C siRNA to silence this phosphatase. Results show that the PP2A/C siRNA efficiently and specifically reduces PP2A/C expression by more than 90%, whereas control siRNA has no effect (Figure 5A). Importantly, the specific silencing of PP2A/C expression results in both enhanced Bcl2 phosphorylation and inhibition of cisplatin-stimulated Bcl2/p53 binding in association with prolonged cell survival (Figure 5). These findings suggest that PP2A may be required for dephosphorylation of Bcl2 and facilitation or enhancement of p53 binding.

PP2A-induced dephosphorylation of Bcl2 results in a conformational change of Bcl2 associated with inactivation of its antiapoptotic function

It is reported that a conformational change in Bcl2 results in the exposure of its BH3 domain in association with inhibition of its antiapoptotic function.[22,25] Because phosphorylation of Bcl2 potently stimulates/facilitates its antiapoptotic function,[11] it is possible that PP2A-mediated Bcl2 dephosphorylation may induce a conformational change leading to inactivation of Bcl2 function. To test this, a Bcl2 BH3 domain-specific antibody was used that can detect a conformational change in the BH3 domain of Bcl2.[25] WT Bcl2-expressing H7 cells were treated with increasing concentrations of C2-ceramide for 24 hours. Immunoprecipitation of Bcl2 was performed using a Bcl2/BH3-domain specific or pan-Bcl2 antibody. Bcl2 was analyzed by Western blotting using a pan-Bcl2 antibody. Results indicate that treatment of cells with C2-ceramide potently enhances the ability of the Bcl2/BH3-domain specific antibody to immunoprecipitate Bcl2 compared with the no treatment control (Figure 6A). To test the specificity of the Bcl2/BH3-domain specific antibody, other BH3-containing proteins (ie, Bax, Bim, and Bad) were also analyzed by Western blot after immunoprecipitation using the Bcl2/BH3 antibody. Results indicate that the Bcl2/BH3 domain-specific antibody does not interact with Bax (Figure 6A) or other BH3-containing proteins (ie, Bim, Bad; data not shown). In addition, the Bcl2/BH3 antibody binding to Bcl2 was confirmed by immunofluorescence. Cells were treated with C2-ceramide (30 μM) for 24 hours, fixed, and permeabilized with ice-cold methanol and acetone. Fixed cells were stained with the Bcl2/BH3 domain primary and FITC-conjugated secondary antibodies. Bcl2 immunofluorescence is low or undetectable in untreated cells, indicating no conformational

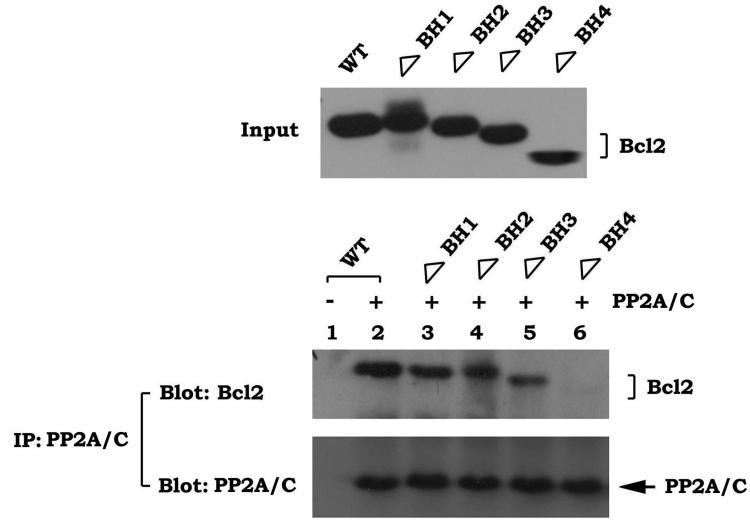

Figure 4. PP2A/C interacts with Bcl2 at its BH4 domain. Purified recombinant PP2A/C was incubated with purified WT, ΔBH1, ΔBH2, ΔBH3, or ΔBH4 Bcl2 deletion mutants in CHAPS lysis buffer at 4°C for 2 hours. The PP2A/C-associated Bcl2 was coimmunoprecipitated with an agarose-conjugated PP2A/C antibody and analyzed by Western blot using a Bcl2 antibody.

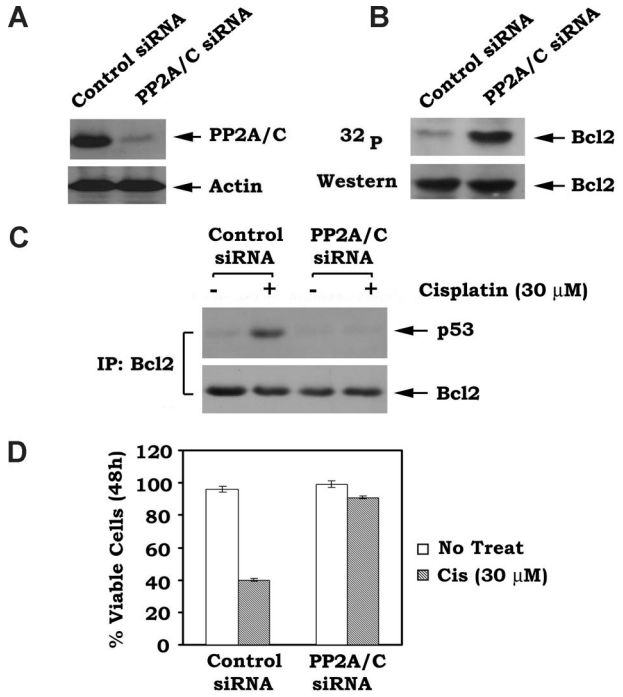

Figure 5. Depletion of PP2A/C by RNAi enhances Bcl2 phosphorylation and suppresses Bcl2/p53 binding in association with increased cell survival. (A) PP2A/C siRNA or control siRNA was transfected into H7 WT Bcl2 cells using Lipofectamine-2000. Expression levels of endogenous PP2A/C were analyzed by Western blot using an anti-PP2A/C antibody. (B) H7 WT Bcl2 cells expressing PP2A/C siRNA or control siRNA were metabolically labeled with ^{32}P-orthophosphoric acid for 2 hours. Phosphorylation of Bcl2 was analyzed by autoradiography. (C) H7 WT Bcl2 cells expressing PP2A/C siRNA or control siRNA were treated with cisplatin (30 μM) for 24 hours followed by lysis in 1% CHAPS-containing buffer. Coimmunoprecipitation was performed using an agarose-conjugated Bcl2 antibody. The Bcl2-associated p53 and Bcl2 were analyzed by Western blotting. (D) H7 WT Bcl2 cells expressing PP2A/C siRNA or control siRNA were treated with cisplatin (30 μM) for 48 hours. Cell viability was determined by analyzing annexin V binding on FACS. Data represent the mean plus or minus SD of 3 separate determinations.

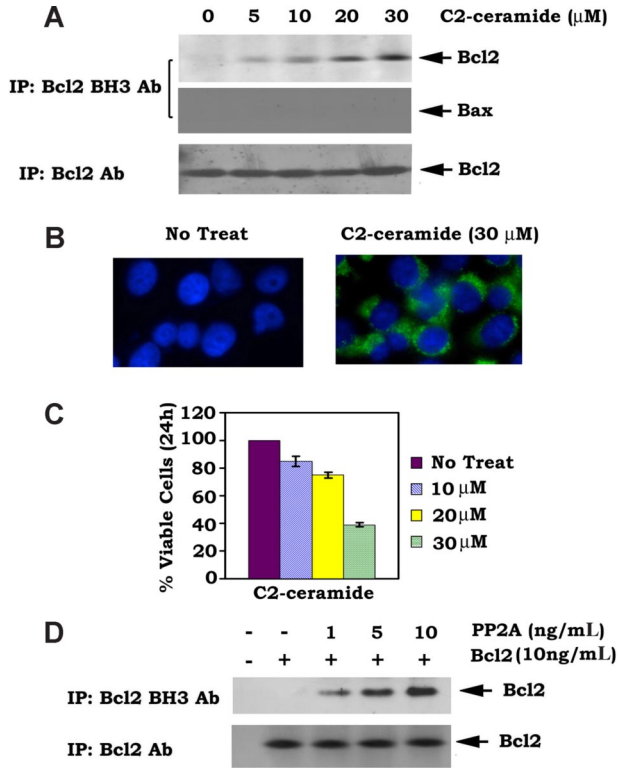

Figure 6. C2-ceramide induces a conformational change of Bcl2 by exposing its BH3 domain in association with apoptosis. (A) H7 WT Bcl2 cells were treated with increasing concentrations of C2-ceramide for 24 hours. Coimmunoprecipitation was carried out using a Bcl2 BH3 or full-length Bcl2 antibody, respectively. Bcl2 or Bax was analyzed by Western blot using a Bcl2 or Bax antibody, respectively. (B) H7 WT Bcl2 cells were treated with C2-ceramide for 24 hours. Cells were then washed with 1× PBS, fixed and permeabilized with ice-cold methanol and acetone, and then blocked with 10% rabbit serum followed by staining with 4,6-diamino-2-phenylindole, a Bcl2 BH3 domain primary, and FITC-conjugated (green) secondary antibodies. Images were merged using Open-lab 3.1.5 software. (C) H7 WT Bcl2 cells were treated with increasing concentrations of C2-ceramide for 24 hours. Cell viability was determined by analyzing annexin V binding on FACS. Data represent the mean plus or minus SD of 3 separate determinations. (D) Purified Bcl2 protein was incubated with increasing concentrations of purified PP2A in 1% CHAPS lysis buffer at 4°C for 2 hours. Coimmunoprecipitation was carried out using a Bcl2 BH3 or full-length Bcl2 antibody, respectively. Bcl2 was analyzed by Western blotting.

change in Bcl2. In contrast, immunofluorescence increases significantly in cells treated with C2-ceramide in association with apoptosis (Figure 6B,C). These findings indicate that ceramide-induced Bcl2 dephosphorylation induces a conformational change in Bcl2, leading to inactivation of its antiapoptotic function. To test whether PP2A can directly induce such a conformational change of Bcl2, an in vitro cell-free assay was used. Intriguingly, addition of purified, active PP2A enhances the ability of the Bcl2/BH3-domain specific antibody to bind Bcl2, which occurs in a PP2A dose-dependent manner (Figure 6D). These findings provide strong evidence that Bcl2 dephosphorylation by PP2A may change Bcl2's conformation.

Discussion

We previously discovered that IL-3 or bryostatin-induced Bcl2 phosphorylation with a peak(s) occurs within 30 minutes but that phosphorylation declines to a baseline level with extended incubation time in IL-3–dependent murine myeloid NSF.H7 cells,[21] indicating its reversible and dynamic nature. Furthermore, OA, a potent inhibitor of PP2A and PP1, not only stimulates but also enhances agonist-induced Bcl2 phosphorylation.[21] We have studied the mechanism for this and found that PP2A functions as a physiologic regulator of Bcl2 because specific disruption of PP2A activity by either expression of small t antigen or depletion of PP2A/C by RNAi results in increased Bcl2 phosphorylation (Figures 1,5). Recent reports reveal that DNA damage can induce p53 translocation to the outer mitochondrial membrane where it may interact with and inactivate Bcl2's antiapoptotic function.[22,26,27] We recently reported that the nonphosphorylatable AAA Bcl2 mutant has an enhanced ability to associate with p53, whereas the hyperphosphorylated, phosphomimetic EEE mutant Bcl2 was much less efficient,[22] suggesting that the phosphorylation status of Bcl2 may regulate Bcl2/p53 binding. Because inhibition of PP2A activity can suppress cisplatin-stimulated Bcl2/p53 binding and prolong cell survival (Figure 1), this indicates that enhanced Bcl2 phosphorylation can negatively regulate p53/Bcl2 interaction. Thus, PP2A-mediated dephosphorylation of Bcl2 facilitates its interaction with p53, which results in suppression of Bcl2's survival activity.

Our previous report also demonstrated that Bcl2 can directly interact with PP2A/C, but the site of this binding on Bcl2 was not known.[21] Now, structure-function studies with Bcl2 deletion mutants reveal that PP2A/C directly binds to Bcl2's BH4 domain (Figure 4). Because only the prosurvival Bcl2 family members possess this conserved BH4 domain, this suggests that the BH4 domain is critical for survival function. Intriguingly, either caspase-mediated cleavage or mutagenic removal of the BH4 domain can

abolish Bcl2's antiapoptotic activity, presumably because of the conversion of Bcl-2 to a multidomain Bax-like death effector,[28,29] indicating that the NH_2-terminal BH4 domain (amino acids 6-31) of Bcl2 is required for its antiapoptotic function.[29-31] Because PP2A directly interacts with Bcl2 at the BH4 region (Figure 4), we propose that the BH4 domain functions as the PP2A "docking site" to potentially "bridge" PP2A to access multiple phosphorylation sites (ie, T69, S70, and S87) located in the flexible loop domain of Bcl2 adjacent to the N-terminal BH4 domain. This mechanism may contribute to removal of Bcl2's phosphate leading to inhibition of its antiapoptotic activity.

The sphingolipid ceramide is well described to function as a powerful second-signal effector molecule that can regulate diverse cellular processes, including apoptosis, cell cycle, cell senescence, cell adhesion, and cellular differentiation.[32,33] Physiologically, hydrophobic ceramide is confined to membranes and probably exerts its effect at the subcellular site of production.[34] Intriguingly, ceramide can stimulate PP2A activity by binding to its catalytic domain.[35,36] It has become clear that ceramide generation is a near-universal feature of apoptosis because ceramide has been implicated in activating both the extrinsic (ie, death receptor) and intrinsic (ie, mitochondrial) apoptotic pathways.[33] However, the signal mechanism(s) by which ceramide may promote apoptosis is not fully understood. We previously discovered that the PP2A activator C2-ceramide can induce Bcl2 dephosphorylation and promote apoptosis.[35] Our results extend this by indicating that C2-ceramide–induced Bcl2 dephosphorylation facilitates/promotes p53/Bcl2 binding in association with inhibition of Bcl2/Bax heterodimerization (Figure 3B). These findings indicate a novel mechanism by which ceramide may induce apoptosis. Both ceramide-stimulated Bcl2 dephosphorylation in vivo or PP2A-induced Bcl2 dephosphorylation in vitro mediate a conformational change in Bcl2 detected by the Bcl2/BH3 antibody that recognizes the inactive form of Bcl2 (Figure 6), suggesting that dephosphorylation of Bcl2 can result in exposure of its BH3 domain, which is normally hidden in the hydrophobic domain of Bcl2. Thus, ceramide-induced Bcl2 dephosphorylation may inactivate Bcl2 via a conformational change in the BH3 domain that then facilitates/promotes Bcl2 and p53 association (Figures 3B, 6). Thus, the dephosphorylated form of Bcl2 induced by ceramide-activated PP2A may more efficiently facilitate formation of the p53/Bcl2 complex. These results reveal a novel mechanism by which dephosphorylation of Bcl2 negatively regulates its antiapoptotic activity.

In conclusion, our findings demonstrate that PP2A is a direct physiologic regulator of Bcl2 that functions by binding to Bcl2's BH4 domain and dephosphorylating and thereby inactivating Bcl2's potent antiapoptotic function. Ceramide, a physiologic death regulator, can activate PP2A to directly dephosphorylate Bcl2, resulting in a conformational change by exposure of its BH3 domain that facilitates formation of the Bcl2/p53 complex in association with apoptotic cell death. Thus, the activation of PP2A with dephosphorylation of Bcl2 that occurs by enhancing ceramide production, in combination with DNA-damaging agent(s), may more potently stimulate p53/Bcl2 binding to result in a synergistically therapeutic effect in hematologic malignancies that express Bcl2.

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Authorship

Contribution: X.D. designed and performed research, analyzed data, and wrote the paper; F.G. performed research; and W.S.M designed research and wrote the paper.

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Correspondence: W. Stratford May, University of Florida Shands Cancer Center, 1376 Mowry Road, Cancer/Genetics Research Complex, Room 363, PO Box 103633, Gainesville, FL 32610-3633; e-mail: smay@ufl.edu.

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