Ceramide Induces Bcl2 Dephosphorylation via a Mechanism Involving Mitochondrial PP2A*

(Received for publication, March 31, 1999, and in revised form, May 14, 1999)

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Phosphorylation of Bcl2 at serine 70 is required for its potent anti-apoptotic function. We have recently shown that Bcl2 phosphorylation is a dynamic process that involves the protein kinase Cα and protein phosphatase 2A (PP2A) (Ruvolo, P. P., Deng, X., Carr, B. K., and May, W. S. (1998) J. Biol. Chem. 273, 25436–25442; and Deng, X., Ito, T., Carr, B. K., Mumby, M. C., and May, W. S. (1998) J. Biol. Chem. 273, 34157–34163). The potent apoptotic agent ceramide can activate a PP2A, suggesting that one potential component of the ceramide-induced death signal may involve the inactivation of Bcl2. Results indicate that C2-ceramide but not inactive C2-dihydroceramide, was found to specifically activate a mitochondrial PP2A, which rapidly and completely induced Bcl2 dephosphorylation and correlated closely with ceramide-induced cell death. Using a genetic approach, the gain-of-function S70E Bcl2 mutation, which mimics phosphorylation, fails to undergo apoptosis even with the addition of high doses of ceramide (IC₅₀ > 50 μM). In contrast, cells overexpressing exogenous wild-type Bcl2 were sensitive to ceramide at dosages where PP2A is fully active and Bcl2 would be expected to be dephosphorylated (IC₅₀ = 14 μM). These findings indicate that in cells expressing functional Bcl2, the mechanism of death action for ceramide may involve, at least in part, a mitochondrial PP2A that dephosphorylates and inactivates Bcl2.

Ceramide, a naturally occurring membrane sphingolipid, has emerged as an important second messenger molecule in apoptosis signaling (1–4). Although the role of glycerophospholipids (e.g. diacylglycerol (DAG)1) in signal transduction has long been known, sphingolipids have only recently been identified as effector molecules (1, 2). Interestingly, DAG and ceramide are closely linked metabolically, and each molecule resides at opposite ends of the sphingomyelin cycle (1). Whereas DAG stimulates cell proliferation and promotes cell survival in many cells, ceramide arrests cell growth and induces programmed cell death (1–4). The opposing effects of DAG and ceramide have therefore led to the hypothesis that one possible mechanism for regulating apoptosis may involve the dynamic cellular generation of these two molecules (1).

Ceramide is known to affect a number of different enzymes involved in diverse signal transduction pathways (5–12). Ceramide has been shown to stimulate the activity of a cytosolic phosphatase, CAPP, which has been identified as a member of the PP2A phosphatase family (5–7). Ceramide also activates a diverse array of kinases including KSR (kinase suppressor of Ras) (9), PKCα (10), and various members of the mitogen- and stress-activated signal transduction pathways (11, 12). Still, the mechanism by which ceramide can induce apoptosis is not known.

A close association between the production of ceramide and the onset of programmed cell death has been well established (13). A number of diverse apoptosis-promoting agents, including tumor necrosis factor α (13), chemotherapeutic drugs (14, 15), ischemia/reperfusion (16), FAS antigen activation (17), irradiation (18), and corticosteroids (19) all can apparently generate ceramide by the induction of sphingomyelin hydrolysis. This phenomena almost suggests that the production of ceramide in response to apoptotic stress stimuli may be an universal element of apoptosis (4). Treatment of cells with water-soluble analogs of ceramide such as C2-ceramide can induce apoptosis, whereas biologically inactive ceramide analogs (e.g., C2-dihydroceramide) have no effect on cell viability (13). Furthermore, recent studies have shown that introduction of bacterial sphingomyelinase in human leukemic cells, to generate intracellular ceramide, results in the induction of apoptosis (20).

Recently, we found that functional phosphorylation of Bcl2 at serine 70 is required for the full and potent anti-apoptotic function of Bcl2 (21, 22). Furthermore, phosphorylation is mediated, at least in part, by mitochondrial PKCα (23) in a dynamic process involving PP2A, a physiologic Bcl2 phosphatase (24). As ceramide can activate PP2A (5–7) and also indirectly inactivate PKCα (25), this raises the possibility that ceramide generation may affect Bcl2 phosphorylation and function. Studies were designed to test the effect of ceramide on Bcl2 phosphorylation and its abilities to suppress apoptosis and to identify a potential mechanism(s) for ceramide-induced Bcl2 dephosphorylation.

**EXPERIMENTAL PROCEDURES**

Materials—All reagents used were purchased from commercial sources unless otherwise stated.

Cell Lines—HL60 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 + 10% bovine calf serum at 37 °C in 5% CO₂. Murine leukemia L1210 cells transfected with WT, S70A, or S70E Bcl2 were maintained as described previously (21, 22).

Analysis of Cell Viability and Apoptosis—Cells were treated with increasing doses of C2-ceramide (Calbiochem) or with C2-dihydroceramide (Calbiochem) for 3, 24, or 48 h. Cell viability was measured by trypan blue dye exclusion, and apoptosis was analyzed using a variation

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of a conventional FACS method to detect sub-2N DNA content in nonviable cells (23).

**Metabolic Labeling, Immunoprecipitation, and Immunoblotting Analysis**—Cells were labeled with [32P]orthophosphoric acid, and Bcl2 was analyzed by immunoprecipitation as described previously (22–24). Where indicated, cells were treated with 1, 10, or 50 μM C2-ceramide or 50 μM C2-dihydroceramide for 3 h. Samples were electrophoresed in a 12% acrylamide, 0.1% SDS gel, transferred to nitrocellulose, and exposed to Kodak X-Omat film at −80 °C. The same blot was used for Western blotting with an anti-Bcl2 antisera or an anti-actin antibody (Sigma) and developed using ECL (Amersham Pharma Biotech kit) as described previously (22).

**Cell Fractionation and Immunolocalization Studies**—Subcellular fractionation of cells was performed as described previously (23). Where appropriate, cells were treated with 10 μM C2-ceramide for 3 h prior to fractionation. Cells were swelled in ice-cold hypotonic Hepes buffer (10 mM Hepes, pH 7.4, 5 mM MgCl2, 40 mM KCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min, aspirated repeatedly through a 25-gauge needle (25 strokes), and centrifuged at 200 × g to pellet the nuclei. The resulting supernatant was centrifuged at 10,000 × g to pellet the heavy membrane fraction containing mitochondria. The purity of the heavy membrane fraction was assessed as described previously (Ref. 23; data not shown).

**Protein Phosphatase Assay**—The protein phosphatase activity of total cell mitochondria and membrane fractions was determined by measuring the generation of free PO4 from the phosphopeptide RRA(pT)VA using the molybdate-malachite green-phosphate complex assay as described by the manufacturer (Promega, Madison, WI). Cell lysates were prepared in a low detergent lysis buffer (0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin), and mitochondrial membranes were prepared as described above. The phosphatase assay was performed in a PP2A-specific reaction buffer (final concentration 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin) using 100 μM phosphopeptide substrate and 1 μg of protein isolated from either HL60 cell lysate or mitochondrial membrane fraction. After a 30-min incubation at room temperature, molybdate dye was added, and free phosphate was measured by optical density at 590 nM. A standard curve with free phosphate was used to determine the amount of free phosphate generated. Phosphatase activity was defined as pmol of free PO4 generated/μg protein/min.

**RESULTS AND DISCUSSION**

It was well established that treatment of HL60 cells with C2-ceramide but not the inactive C2-dihydroceramide analog induces cells to undergo apoptosis (13). HL60 cells were treated with varying doses of C2-ceramide and C2-dihydroceramide for 24 h to assess cell viability (Fig. 1A). Consistent with recent findings (13, 19), the inactive C2-dihydroceramide analog had no effect on HL60 cell survival, but cells were killed by C2-ceramide (i.e. IC50 = 5.3 μg, Fig. 1A). The mechanism of cell death was determined to be apoptosis by detection of apoptotic cell populations that display a sub-2N genomic content during FACS sorting (data not shown; 23).

We recently discovered that Bcl2 phosphorylation at serine 70 is required for the full and potent suppression of apoptosis (21, 22). Phosphorylation is mediated, at least in part, by mitochondrial PKC α (23). HL60 cells express abundant levels of mitochondrial PKC α and have robust basal levels of phosphorylated Bcl2 (23). To determine the effect of ceramide on Bcl2 phosphorylation, HL60 cells were treated with varying doses of C2-ceramide during metabolic labeling with [32P]orthophosphosphate, and the phosphorylation status of Bcl2 was examined following immunoprecipitation. Western analysis demonstrates that roughly equivalent levels of Bcl2 protein were immunoprecipitated from control and C2-ceramide-treated samples. However, even though Bcl2 is highly phosphorylated in HL60 cells, its phosphorylation status is inhibited in a dose-dependent manner when the cells are treated with increasing doses of C2-ceramide (Fig. 1B). Furthermore, ceramide-induced Bcl2 dephosphorylation closely correlates with reduced survival. For example, 1 μM C2-ceramide has little effect on either HL60 cell survival or Bcl2 phosphorylation, whereas increasing doses of C2-ceramide promote Bcl2 dephosphorylation and result in decreased survival (Fig. 1, A and B). By contrast, the addition of the inactive C2-dihydroceramide, even at a high concentration (50 μM), has no such effect on either Bcl2 phosphorylation or survival (Fig. 1, A and B). These findings suggest that Bcl2 phosphorylation can be inhibited by active ceramide in a mechanism that may involve a Bcl2 phosphatase. Interestingly, ceramide-induced dephosphorylation of Bcl2 can be blocked by the potent phosphatase inhibitor okadaic acid (Fig. 1C), suggesting a mechanism involving an okadaic acid-sensitive phosphatase. Because PP2A but not PP1 is a Bcl2 phosphatase (24) and ceramide has been reported to activate a cytosolic PP2A (5–7), a potential mechanistic link exists. To test this possibility, the effect of ceramide treatment on PP2A activity in HL60 cells was assessed. Cells were treated with varying doses of C2-ceramide for 3 h and lyzed in a low detergent lysate buffer (see “Experimental Procedures”). The phosphatase activity in protein lysates was determined by measuring the generation of free phosphate liberated from a phosphopetide substrate, RRA(pT)VA, which can be specifically recognized by PP2A, PP2B, or PP2C depending on the phosphatase buffer conditions used (26). Unlike PP2B or PP2C, PP2A activity does not require any cations and can be specifically assessed when both Ca2+ and Mg2+ are eliminated from the buffer (27). Under these conditions, results indicate that C2-ceramide stimulates phosphatase activity in a dose-dependent manner (Fig. 2A). Importantly, low doses of C2-ceramide (1 μM) only minimally stimulate (~1.4-fold) activity, whereas higher concentrations of C2-ceramide (>10 μM) potently increase activity (~2.5-fold; Fig. 2A). The phosphatase activity observed is likely the result of PP2A or a PP2A-like phosphatase, because inclusion of 10 nM okadaic acid directly in the assay buffer results in >90% inhibition of the phosphatase activity (data not shown).

Although this possibility is intriguing, it cannot be ruled out that stimulation of PP2A phosphatase activity merely reflects the activation of cytosolic CAPP, which may not directly affect phosphorylation of mitochondrial Bcl2. If ceramide is indeed affecting a Bcl2 phosphatase, it is expected that activation will occur at the mitochondrial membrane level. To test this possibility, the effect of C2-ceramide on PP2A activity in mitochondrial membranes from HL60 cells was determined. Following treatment with 10 μM C2-ceramide for 3 h, mitochondrial membranes were isolated as described previously (23, 24). Results indicate that C2-ceramide stimulates a greater than 5-fold increase in PP2A activity, whereas cells treated with the inactive C2-dihydroceramide analog demonstrate no stimulation of mitochondrial PP2A (Fig. 2B). Moreover, Fig. 2B shows that pretreatment of cells with 100 nM okadaic acid can totally prevent stimulation of phosphatase activity by C2-ceramide. Therefore, we conclude that C2-ceramide-induced Bcl2 dephosphorylation may result from the activation of a mitochondrial PP2A.

The question then remains whether Bcl2 dephosphorylation is required for ceramide-induced apoptosis in cells expressing Bcl2. It has recently been reported that interleukin-3-dependent murine myeloid cells that overexpress exogenous wild-type (WT) Bcl2 are protected from rapid apoptosis induced by growth factor withdrawal or chemotherapeutic drugs (21, 22). Furthermore, the nonphosphorylatable, loss-of-function serine
70 to alanine (S70A) mutant Bcl2 fails, however, to fully suppress apoptosis (22). By contrast, a serine 70 to glutamate (S70E) Bcl2 mutant, which may mimic a phosphorylation charge, is able to efficiently suppress apoptosis, indicating a gain-of-function mutation (22). Therefore, the effect of increasing concentrations of ceramide on the Bcl2 mutants was tested. The data presented are representative of at least three clones tested for each transfectant that was previously shown to be phenotypically similar (22). Each transfectant demonstrates a 6–8-fold increase above the endogenous level of Bcl2 (22). The S70E Bcl2 and S70 A Bcl2 transfectants express roughly equivalent levels of Bcl2 protein, whereas the WT Bcl2 transfectant expresses roughly 30% more protein than the other transfectants as determined by densitometry (Fig. 3). This finding suggests that any observed effects are unlikely because of differences in Bcl2 expression. Results indicate that the S70A Bcl2 mutant fails to protect cells from C2-ceramide induced apoptosis, whereas cells expressing exogenous WT Bcl2 are protected from low (i.e. < 10 μM) but not high concentrations of C2-ceramide, which potently activates PP2A (Fig. 3, B and C). Interestingly, after 48 h in the presence of ceramide, cells expressing WT Bcl2 become sensitive to C2-ceramide, whereas the S70E Bcl2 mutant remains potently able to protect cells from apoptosis (Fig. 3, B and C). These data strongly support a mechanism by which serine 70 phosphorylation of Bcl2 is required to prevent ceramide-induced cell death in interleukin-3-dependent myeloid cells. Whereas increasing concentrations of ceramide can increase both PP2A activation and Bcl2 dephosphorylation, the S70E Bcl2 mutant would be expected to be insensitive to PP2A, and thus the cells would remain resistant to ceramide-induced apoptosis.

A previous study has shown that overexpression of exogenous Bcl2 can protect cells from ceramide-induced cell death (14). Although this study did not address the phosphorylation status of Bcl2, the ceramide concentrations tested did not exceed 5 μM (14). Thus, exogenous Bcl2 may have undergone only incomplete dephosphorylation during the treatment time of cells, which could have allowed at least some protection. It is
therefore interesting to speculate that ceramide may somehow suppress cell anti-apoptotic pathways involving Bcl2. Because most cell types are sensitive to ceramide but not all cells express Bcl2 (28), it would be important to determine the effect of ceramide on other Bcl2 family members. A recent study has shown that ceramide can block Bad phosphorylation through inactivation of the Bad kinase, Akt, and thus promote the pro-apoptotic function of Bad (29). The mechanism by which ceramide was thought to function in this system was concluded to be independent of a PP2A-like phosphatase because treatment of cells with okadaic acid failed to reverse ceramide-induced inactivation of Akt (29, 30). It thus appears that ceramide may have an inhibitory effect on Bcl2 family members by disrupting regulatory phosphorylation. Thus, ceramide can apparently have a dual role in promoting the pro-apoptotic activity of Bad while simultaneously blocking the anti-apoptotic function of Bcl2. Such an effect may have a profound additive or synergistic effect on apoptosis.

The production of ceramide is so common during apoptosis induced by diverse stress stimuli (15–18) that it has been considered a universal feature of this process (1, 4). It has been suggested that ceramide and diacylglycerol, two potent effectors situated at opposite ends of the sphingomyelin cycle, may have counterbalancing effects on cell growth and apoptosis (1, 31). At least one enzyme controlling the sphingomyelin cycle, sphingomyelin synthase, has been suggested to simultaneously regulate DAG and ceramide levels but in opposite directions (31). In addition, DAG has been shown to suppress ceramide-induced apoptosis (32). In support of this possibility, cells that are naturally resistant to FAS ligand-induced (17) or IgM-induced (18) apoptosis exhibit only very low levels of ceramide, whereas the addition of ceramide to these cells restores FAS sensitivity (17). Thus, it would appear logical that ceramide might function in a mechanism that features dephosphorylation and inactivation of Bcl2. Because serine 70 Bcl2 phosphorylation is necessary to block ceramide-induced apoptosis, it is possible that this post-translational modification enables Bcl2 to act as a stress “sensor” molecule. Fig. 4 depicts a model that illustrates how differential levels of DAG and ceramide may affect Bcl2 phosphorylation and thus influence its survival function. Stress conditions, such as those induced by tumor necrosis factor α (13), chemotherapeutic drugs (14, 15), ischemia/reperfusion (16), FAS antigen activation (17), irradiation (18), and corticosteroids (19), may favor ceramide generation by the activation of sphingomyelinase and ceramide synthase (1, 4). The resulting increased cellular ceramide levels could stimulate PP2A, a physiologic Bcl2 phosphatase, to dephosphoryl-
Growth and Survival

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\text{DAG} \xrightarrow{\text{Sphingomyelin Synthase}} \text{Ceramide}
\]

Bcl2

DAG \xrightarrow{\text{PKC}} \text{Ceramide Synthase} \xrightarrow{\text{Sphingomyelinase}} \text{Ceramide}

PKCα

Ser70 -PO₄

Growth Arrest and Death

PKCα

PP2A

Ser70

Ceramide

Non-functional Bcl2

Functional Bcl2

FIG. 4. Bcl2 phosphorylation status could be influenced by DAG and ceramide levels. A model is presented in which Bcl2 function is regulated by effector molecules. Sphingomyelin synthase promotes DAG production at the expense of ceramide during non-stress conditions. Conversely, stress conditions induce ceramide production at the expense of DAG during non-stress conditions. Conversely, stress conditions promote ceramide production and inactivate Bcl2. Conversely, diacylglycerol can not only promote cell growth and survival in many systems (33), but can also activate a physiologic Bcl2 kinase, PKCα, to support cell survival (23). Understanding how apoptosis may be differentially regulated by effector/secondary messenger molecules may provide insight into developing novel clinical strategies aimed at defeating chemoresistance in cancer cells protected by Bcl2.

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