A Functional Role for the B56 α-Subunit of Protein Phosphatase 2A in Ceramide-mediated Regulation of Bcl2 Phosphorylation Status and Function*

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Recently it has been shown that the potent apoptotic agent ceramide activates a mitochondrial protein phosphatase 2A (PP2A) and promotes dephosphorylation of the anti-apoptotic molecule Bcl2 (Ruvolo, P. P., Deng, X., Ho, T., Carr, B. K., and May, W. S. (1999) J. Biol. Chem. 274, 20296–20300). In cells expressing Bcl2, dephosphorylation of Bcl2 appears to be required for ceramide-induced cell death because treatment of cells with low doses of the PP2A inhibitor okadaic acid blocks Bcl2 dephosphorylation and promotes cell survival. Furthermore, the non-phosphorylatable (i.e. PP2A-resistant) gain-of-function S70E mutant Bcl2 can protect cells from ceramide-induced apoptosis. These findings support a model whereby Bcl2 function is regulated by PP2A. PP2A is a heterotrimer that contains a catalytic C-subunit, a structural A-subunit, and a regulatory B-subunit. The A- and C-subunits are fairly conserved and ubiquitously expressed, and they form the catalytic complex of the phosphatase. In contrast, there are at least three families of diverse B-subunit molecules that vary in expression temporally and by tissue type. It is hypothesized that ceramide regulates PP2A via the B-subunit. Thus, understanding the mechanism of how PP2A regulates Bcl2 phosphorylation status and how ceramide might regulate this process requires identification of the regulatory B-subunit of PP2A that comprises the Bcl2 phosphatase. Results indicate that the B56 α-subunit is a candidate regulatory subunit of the physiologic Bcl2 phosphatase since (a) B56 α associates with Bcl2 as evidenced by pull-down experiments, (b) B56 α co-localizes with Bcl2 in mitochondrial membranes, (c) ceramide promotes translocation of B56 α to mitochondrial membranes, and (d) overexpression of B56 α promotes mitochondrial PP2A activity and Bcl2 dephosphorylation and potentiates cell killing with ceramide. These findings suggest a role for B56 α in regulating the Bcl2 phosphatase.

The phosphorylation status of Bcl2 has been shown to influence the function of this important anti-apoptotic molecule (1–8). Growth agonist-induced monosite phosphorylation of Bcl2 at serine 70 is required for full and potent anti-apoptotic function of Bcl2 and thus promotes cell survival (1–4). Conversely, treatment of cells with antimitotic agents such as paclitaxel induces multisite phosphorylation of Bcl2 involving both serine and threonine residues and promotes cell death (5–8). Considering the complex nature of the mechanisms regulating Bcl2 function, it is not surprising that a number of protein kinases have been identified as physiologic Bcl2 kinases including protein kinase C (1), ERK1/2 (2), and c-Jun N-terminal kinase (8–10). It is now evident that Bcl2 phosphorylation is a dynamic process that is reversible under growth agonist conditions. The serine/threonine protein phosphatase PP2A1 has been found to have a role in this process (11). It has recently been discovered that ceramide activates a mitochondrial PP2A and can regulate apoptosis by a mechanism involving dephosphorylation of Bcl2 (12). The non-phosphorylatable (i.e. PP2A-resistant) gain-of-function S70E mutant Bcl2 can protect cells from ceramide-induced apoptosis at concentrations of ceramide (i.e. >10 μM) where wild-type Bcl2 is not phosphorylated and fails to protect cells from ceramide-induced killing (12). This finding strongly suggests that dephosphorylation of Bcl2 is required for ceramide-induced cell death in cells expressing Bcl2. Because ceramide production is a nearly universal component of apoptosis (13–16), it is possible that such a mechanism may exist in response to other apoptotic stimuli (17).

Identification of the PP2A isoform(s) that is the physiologic Bcl2 phosphatase will be critical for determining the mechanism by which PP2A regulates Bcl2 function. PP2A is a major protein serine/threonine phosphatase that participates in many signaling pathways in mammalian cells (18). It is a heterotrimer consisting of a catalytic subunit (the 36-kDa C-subunit PP2A/C), a structural subunit (the 65-kDa A-subunit PP2A/A), and a regulatory subunit (the B-subunit PP2A/B, which can vary in size from 50 to 130 kDa). The catalytic and structural subunits are evolutionarily conserved (18–21). There are two highly homologous isoforms each of the C-subunit (98% amino acid homology between α and β isoforms) (18–20) and the A-subunit (87% amino acid homology between α and β isoforms) (12, 15). The A- and C-subunits are ubiquitously expressed (22), and they form a catalytic complex (PP2A/AC) that interacts with at least three families of regulatory subunits (B, B56, and PR72/130) and tumor antigens (e.g. SV40 small T antigen) (18).

The regulatory B-subunits of PP2A are expressed differen-
tially by tissue and temporally during development (18, 23–26). In addition, PP2A substrate specificity appears to be determined by the B-subunit (27). Finally, there is evidence that the regulatory B-subunits may target the PP2A catalytic complex to intracellular sites such as microtubules (28) or the nucleus (29). These features of the PP2A regulatory B-subunits suggest that it is the B-subunit that defines PP2A isoforms and their physiologic roles. Thus, identification of the B-subunit(s) that makes up the physiologic Bcl2 phosphatase will elucidate the mechanism by which PP2A regulates Bcl2 function.

**EXPERIMENTAL PROCEDURES**

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

**Cell Lines, Plasmids, and Transfections**—REH and HL60 cells were obtained from the ATCC (Manassas, VA) and maintained in RPMI 1640 + 10% bovine calf serum at 37 °C in 5% CO_2_. The B56α cDNA-containing pCMV plasmid was introduced into HL60 cells by electroporation (200-V, 975-microfarad capacitance) and selected and maintained in the above medium plus 0.6 mg/ml G418 (Invitrogen).

**Analysis of Cell Viability and Apoptosis**—Cells were treated with increasing doses of C2-ceramide (Calbiochem) or etoposide (Sigma) for 24 h. Cell viability was measured by trypan blue dye exclusion, and apoptosis was analyzed using a DNA laddering method as described previously (30).

**Metabolic Labeling,Immunoblotting, and Immunostaining Analysis**—Cells were labeled with [32P]orthophosphoric acid, and the phosphorylation status of Bcl2 was determined by immunoprecipitation as described previously (1). 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 using an anti-Bcl2 antiserum (DAKO, Carpinteria, CA) and developed using ECL (Amersham Biosciences) as described previously (1).

**Cell Fractionation and Immunolocalization Studies**—Subcellular fractionation of cells was performed as described previously (1). Where appropriate, cells were treated with 25 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 MgCl_2, 40 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml apropin, 10 μg/ml leupeptin) for 30 min, aspirated repeatedly through a 25-gauge needle (25 strokes), and centrifuged at 100 × g to pellet nuclei. The resulting supernatant was centrifuged at 10,000 × g to pellet the heavy membrane fraction containing mitochondria. Western blotting was performed as described previously (1) using antibodies to Bcl2 (DAKO), prohibitin (Research Diagnostics Inc., Flanders, NJ), and the following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA): anti-B56α, -PP2A/A, -PP2A/C, and -actin.

**Protein Phosphatase Assay**—Protein phosphatase activity of mitochondrial membran fractions was determined as described previously (12). Mitochondrial membranes (12 mg protein) from the phosophopeptide RAIP1/RAIP2 (where pT is a phosphorylated threonine) was measured using the molydbdate-malachite green-phosphate complex assay as described by the manufacturer (Promega, Madison, WI). Mitochondrial membranes were prepared as described above. The phosphatase assay was performed in the PP2A-specific reaction buffer (final concentration: 50 mM imidazole (pH 7.2), 0.2 mM EDTA, 0.02% 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin) using 100 μg of the phosphopeptide substrate and 2 μg of protein isolated from the 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 pmoles of free PO_4 generated/μg of protein/min.

**Immunohistochemistry Studies**—Cells were treated with methanol to permeabilize and fix the cells. Fixed cells were adhered to a microscope slide using polyethyleneimine. The cells were washed and blocked with sera from the host animal of the secondary antibody to be used, and then primary antibody was added. Finally, an Alexa Fluor-conjugated secondary antibody was added, and cells were visualized using a Zeiss Axiosplan 2 fluorescence microscope and photographed with a Hammamatsu digital camera in black and white. False color, deconvolution and enhancement were accomplished with OpenLab 3.0.4 and Photoshop 6.0. The B56α antibody and PP2A/A antibody are both goat polyclonal sera from Santa Cruz Biotechnology. The Bcl2 antibody used is the mouse monoclonal antibody from DAKO. Alexa Fluor 488-labeled anti-goat serum and Alexa Fluor 594-labeled anti-mouse serum (both from Molecular Probes) were used. Alexa Fluor 488 appears green, and Alexa Fluor 594 appears red when observed using a fluorescent microscope. To determine subcellular regions of protein co-localization, individual red- and green-stained images derived from the same field were merged using Photoshop 6.0. Areas of protein co-localization appear yellow.

**RESULTS**

**B56α Associates with Bcl2**—B56α is up-regulated by ceramide in HL60 cells, although other B regulatory subunit genes are not. If B56α is part of the Bcl2 phosphatase, up-regulation of this PP2A-subunit may explain one way in which ceramide might induce dephosphorylation of Bcl2 in HL60 cells (12). In a model where B56α is the active B-subunit of the physiologic Bcl2 phosphatase, one would predict that B56α would associate with Bcl2. Other subunits of the PP2A heterotrimer have been shown to associate with Bcl2 (11). Both the structural A-subunit and catalytic C-subunits of PP2A are co-immunoprecipitated with Bcl2 in pull-down experiments (11). To determine whether B56α co-immunoprecipitates with Bcl2, pull-down experiments using the Bcl2 antibody were performed. REH cells were used because these cells appear to have high levels of the Bcl2 phosphatase. Despite expressing high levels of Bcl2 protein, little if any Bcl2 is phosphorylated in REH cells (1). The PP2A inhibitor okadaic acid, however, induces phosphorylation of Bcl2 in REH cells, suggesting that PP2A is at least in part responsible for the hypophosphorylated status of Bcl2 in these cells. As shown in Fig. 1, B56α but not Bα/β co-immunoprecipitates with Bcl2 using Bcl2 antisera. Both B-subunit proteins are present in the total lysate of REH cells (Fig. 1). Thus, B56α is a candidate B-subunit comprising the physiologic Bcl2 phosphatase.

Ceramide Promotes B56α Translocation to the Mitochondrial Membranes—A possible mechanism for how ceramide might activate the Bcl2 phosphatase is by promoting the translocation of PP2A to the mitochondrial membranes where the protein phosphatase would be expected to dephosphorylate Bcl2. Because the regulatory B-subunit has been implicated in modulating PP2A subcellular localization (28, 29), it is possible that B56α might recruit PP2A to subcellular locations where Bcl2 is functional. Although Bcl2 sometimes is found in nuclear membranes and in the endoplasmic reticulum (31, 32), there is strong evidence that suppression of apoptosis by Bcl2 requires mitochondrial localization (33). Only Bcl2 that is targeted to mitochondrial membranes can efficiently suppress apoptosis following serum deprivation in both Madin-Darby canine kidney cells and Rat-1/Myct cells (34). Furthermore, the majority of Bcl2 found in REH and HL60 cells is located in the mitochondrial membranes (1, 12). If ceramide activates the Bcl2 phosphatase via mitochondrial PP2A translocation, we would expect B56α to translocate to the mitochondrial membranes in response to ceramide. Immunofluorescence was used to examine the effect of ceramide on B56α co-localization with Bcl2 in REH cells (Fig. 2). REH cells express basal levels of B56α, whereas HL60 cells only express B56α after ceramide treat-

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Role for B56α in Bcl2 Dephosphorylation

**Fig. 2. Ceramide promotes co-localization of B56α with Bcl2.** REH cells were untreated (top) or treated for 1 h with either 25 μM dihydrocERAme (middle) or 10 μM C2-ceramide (bottom). Cells were fixed with methanol. Next, goat B56α polyclonal antibody and mouse monoclonal Bcl2 antibody were added. Fluorescent conjugated secondary antibodies were used to visualize Bcl2 (red) and B56α (green) localization patterns under a fluorescent microscope, and image analysis was performed as described under “Experimental Procedures.” Red- and green-stained images were merged using Photoshop 6.0. Areas of co-localization appear yellow.

**Fig. 3. Ceramide promotes co-localization of PP2A/A with Bcl2.** REH cells that were untreated or treated with 10 μM ceramide for 1 h were fixed with methanol, and then goat PP2A/A polyclonal antibody or mouse monoclonal Bcl2 antibody was added. Fluorescent conjugated secondary antibody was used to visualize PP2A/A (green) and Bcl2 (red) localization patterns using a fluorescent microscope. Image analysis was performed as described in the legend to Fig. 2. Areas of co-localization appear yellow.

ment. REH cells were treated with 25 μM dihydroniceramide or 10 μM C2-ceramide for 1 h. Subcellular distribution of B56α and Bcl2 was then examined by immunofluorescence and compared with untreated cells (Fig. 2). A mouse monoclonal antibody against human Bcl2 (DAKO) and a goat polyclonal B56α antibody (Santa Cruz) was used so that cells could be simultaneously stained, and co-localization was examined using Alexa Fluor 488-labeled anti-goat serum and Alexa Fluor 594-labeled anti-mouse serum (both from Molecular Probes). Alexa Fluor 488 appears green, and Alexa Fluor 594 appears red when observed using a fluorescent microscope. As shown in Fig. 2, the majority of Bcl2 is non-cytosolic because it is distributed in small organelles (presumably mitochondria) as exhibited by the sharp punctate staining pattern, and ceramide has no effect on the subcellular distribution of Bcl2. The inactive ceramide analog, dihydroniceramide, had no effect on subcellular localization of either B56α or Bcl2 (Fig. 2). As seen in Fig. 2, B56α is still localized primarily in the cytosol in the presence of dihydroniceramide. Although the majority of B56α is cytosolic in untreated cells, ceramide promotes co-localization of B56α with Bcl2. B56α in untreated cells displays a diffuse staining pattern, however, after ceramide treatment, and the staining pattern of B56α suggests a movement from the cytosol to organelles as the staining pattern becomes less diffuse and more punctate (Fig. 2). Furthermore, ceramide appears to promote a fair degree of co-localization with Bcl2 as evidenced by merging optical fields stained with both Bcl2 (red) and B56α (green) antibodies. As seen in Fig. 2, ceramide-treated cells display significant regions of the cell that are stained yellow. The yellow staining pattern represents areas where Bcl2 and B56α are co-localized. In contrast, merged images of untreated cells or cells treated with dihydroniceramide display significantly less yellow staining (Fig. 2).

To determine the effect of ceramide on translocation of the PP2A catalytic complex (A- and C-subunits), we examined the effect of ceramide on subcellular distribution of the structural A-subunit (PP2A/A) in REH cells. Similar to B56α, the majority of PP2A/A was cytosolic in untreated cells; however, after ceramide treatment the cells showed a more punctate pattern of PP2A/A staining (Fig. 3). As observed in Fig. 3, there was significantly more co-localization of Bcl2 and PP2A/A in ceramide-treated cells (as evidenced by the significant increase in the yellow staining pattern) compared with untreated cells. This finding suggests that ceramide promotes translocation of the PP2A catalytic complex, possibly through B56α in the case of the Bcl2 phosphatase.

To confirm the results obtained by immunofluorescence, subcellular fractions were isolated as described previously (12). The subcellular fractions include heavy membrane, light membrane, cytosol, and nuclear membrane fractions. The heavy membrane fraction contains mitochondrial membranes and is the predominant subcellular locale for Bcl2 in REH and HL60 cells (1, 12). Prohibitin, a mitochondrial protein, was used as a control (Fig. 4). A nuclear contaminant of prohibitin was observed and likely represented protein from undisrupted cells that were spun down with nuclei. No prohibitin was found in light membrane or cytosolic fractions, which suggests that these fractions are fairly free of mitochondrial membrane proteins.

Prior to ceramide treatment, B56α was found in the heavy membrane and light membrane fractions as well as in the cytosol (Fig. 4). It is not surprising that there was some basal level of B56α existing in the heavy membrane fraction that contained mitochondrial membranes because REH cells exhibited little if any basal level of phosphorylated Bcl2 (1). As shown in Fig. 4, after ceramide treatment cells showed significantly more B56α in the heavy membrane fraction compared with untreated cells. These data suggest that ceramide promotes translocation of B56α from the cytosol to the mitochondrial membrane fraction and support the findings by immunofluorescence that ceramide promotes greater co-localization of B56α with Bcl2. REH cells that were untreated or treated with 10 μM ceramide for 1 h were fixed with methanol, and then goat PP2A/A polyclonal antibody or mouse monoclonal Bcl2 antibody was added. Fluorescent conjugated secondary antibody was used to visualize PP2A/A (green) and Bcl2 (red) localization patterns using a fluorescent microscope. Image analysis was performed as described in the legend to Fig. 2. Areas of co-localization appear yellow.
B56 α protein in untreated cells is cytosolic.

A possible mechanism to explain how ceramide might promote B56 α mitochondrial translocation may involve increased expression of the protein in response to ceramide. As noted earlier, B56 α is up-regulated by ceramide in HL60 cells.² As shown in Fig. 5, REH cells treated with 10 μM C2-ceramide for 3 h expressed more B56 α compared with untreated cells although there was no difference in the expression of actin. Interestingly, ceramide treatment had no effect on expression of PP2A/A (Fig. 5). Simple overexpression of B56 α cannot completely explain ceramide-mediated translocation of the protein to the mitochondrial membrane. Although there was clearly more B56 α in the heavy membrane fraction of ceramide-treated cells (as displayed in Fig. 4), there appears to be little difference in the amount of B56 α found in light membrane fractions of treated compared with untreated cells. Still, it will be important to determine the exact mechanism by which ceramide promotes B56 α mitochondrial translocation.

We have demonstrated previously that although HL60 cells exhibit little (if any) mitochondrial PP2A activity, ceramide robustly promotes mitochondrial PP2A activity in HL60 cells (12). To determine whether ceramide promotes mitochondrial localization of PP2A, HL60 cells were treated with ceramide, and PP2A expression was observed in subcellular fractions as described above (Fig. 4). In untreated HL60 cells little (if any) PP2A/A was found co-localized with Bcl2 in the mitochondrial membrane-containing heavy membrane fraction (Fig. 6). Fig. 6 demonstrates that ceramide has little effect on Bcl2 subcellular localization. However, treatment of HL60 cells with C2-ceramide promoted localization of PP2A/C to mitochondrial membranes. The level of PP2A/C in the heavy membrane fraction of ceramide-treated cells was more than twice that of untreated cells relative to the level of mitochondrial Bcl2 as determined by densitometry of the respective bands in Fig. 6. Ceramide-induced translocation of PP2A to the mitochondrial membranes was consistent with the promotion of mitochondrial PP2A activity by ceramide as has been reported recently (12). HL60 cells treated with ceramide showed a roughly 7-fold increase in PP2A activity in isolated mitochondrial membranes (12).

**Overexpression of Exogenous B56 α Promotes Mitochondrial PP2A Activity, Bcl2 Dephosphorylation, and Chemosensitivity in HL60 Cells**—The B56 α-subunit gene under the cytomegalovirus promoter was stably transfected into HL60 cells. Introduction of exogenous B56 α did not affect the viability of HL60 cells (data not shown). Two transfectants were obtained (i.e., clone 3 and clone 9), and both clones demonstrated higher levels of B56 α as observed in Western blot analysis of total protein lysates (Fig. 7). Overexpression of B56 α resulted in greater mitochondrial PP2A activity (Fig. 8). Subcellular fractions containing mitochondrial membranes were isolated, and in vitro PP2A assays were performed as described previously (12). Cells expressing exogenous B56 α displayed almost twice the basal level of mitochondrial PP2A activity compared with parental HL60 cells as shown in Fig. 8.

Introduction of B56 α into HL60 cells resulted in reduced levels of basal Bcl2 phosphorylation (compare untreated parent and clone samples in Fig. 9). Furthermore, as shown in Fig. 9, Bcl2 dephosphorylation was accelerated by ceramide in HL60 cells expressing exogenous B56 α. This finding suggests that B56 α overexpression promotes activity of the Bcl2 phosphatase in a ceramide-responsive manner. Consistent with previ-
ous findings correlating Bcl2 phosphorylation status and sensitivity to ceramide in HL60 cells (12), the B56 α transfectants were more sensitive to stress induced by ceramide treatment compared with parental cells. HL60 cells overexpressing B56 α were ∼9× more sensitive to ceramide than HL60 parental cells (Fig. 10). The IC_{50} for ceramide in parental HL60 cells was approximately 18 μM whereas clone 9 B56 α transfectant cells displayed an IC_{50} of 2 μM as derived from the curve in Fig. 10. In addition, B56 α transfectant cells were at least 3× more sensitive to the chemotherapeutic drug etoposide (Fig. 7). The IC_{50} value for parental HL60 cells treated with etoposide (after 24 h) was 14 μM, whereas clone 3 and clone 9 demonstrated IC_{50} values of 4 and 1 μM, respectively. These results strongly suggest that overexpression of B56 α in HL60 cells promotes mitochondrial PP2A activity resulting in reduced levels of phosphorylated (i.e. functional) Bcl2, which leads to greater chemosensitivity.

DISCUSSION

PP2A is a heterotrimer composed of a catalytic subunit (C-subunit), a structural subunit (A-subunit), and a regulatory subunit (B-subunit) (18). The B-subunit best defines each specific PP2A isoform because it is the most diverse subunit (18, 26). Although there are only two variants each of the A- and C-subunits, there are at least three families of B-subunits (B, B56, and PR72/130) that are each composed of multiple isoforms (18–23, 25, 26). In addition, the B-subunit determines PP2A specificity and therefore defines the PP2A substrate target (27–29). It is likely, however, that physiologic specificity of the target depends more on PP2A localization than on contributions from the B-subunit because the AC catalytic complex of PP2A alone has phosphatase activity (18). It is possible that the regulatory B-subunit recruits PP2A catalytic core complex to sites of activity (28, 29). The B α-subunit is involved in translocation of PP2A to microtubules (28), whereas members of the B56 family are involved in nuclear translocation (29). It is yet to be determined whether B-subunit molecules chaperone the PP2A/AC catalytic core to the mitochondria. However, findings reported here suggest that B56 α promotes mitochondrial PP2A activity, suggesting that perhaps mitochondrial PP2A activity is mediated by this B-subunit (Fig. 8). Thus, it is possible that PP2A substrates may be targeted by localization of the enzyme via the B-subunit. There is evidence that PP2A regulatory B-subunits are potential ceramide targets (35, 36), thus suggesting that ceramide may activate PP2A via the regulatory B-subunit. Data presented here demonstrate that ceramide induces PP2A/A (Figs. 3 and 6) and B56 α (Figs. 2 and 4) translocation to mitochondrial membranes where Bcl2 is present. Interestingly, the various B-subunit family members, despite a lack of sequence similarity between the families, recognize similar segments of the structural A-subunit of PP2A (37). The regulation of PP2A activity by the regulatory B-subunits is thus more complicated than binding to the PP2A catalytic complex. Recent studies have shown that post-translational modification of PP2A results in both positive and negative regulation of the enzyme (37). Tyrosine phosphorylation of the catalytic C-subunit results in PP2A inactivation (38). On the other hand, serine phosphorylation of at least one B-subunit (B56 α) appears to promote PP2A activity (39). Most of the members of the B56 regulatory subunit family (with the exception of the γ1 isoform) are phosphoproteins (29). Phosphorylation of B56 α has been shown to promote in vivo PP2A activity, at least where eukaryotic initiation factor 4E is the substrate (39). How phosphorylation of B56 proteins may affect PP2A activity is not clear. Interestingly, phosphorylation of the B56 proteins does not affect their ability to bind the catalytic AC complex (29). Although phosphorylation of B56 α has been shown to promote in vitro phosphatase activity (39), the effect of phosphorylation of B56 proteins on subcellular localization has not been investigated. It is possible that phosphorylation of the various B56 family members promotes translocation. The concept that ceramide can promote mitochondrial translocation of B56 α (see Fig. 2) represents indirect evidence for this notion. It is possible that ceramide promotes phosphorylation of B56 α. Ceramide has recently been found to activate the dsRNA-dependent protein kinase PKR (30). Importantly, PKR recently has been shown to be a B56 α kinase (39). Thus, ceramide may activate PKR resulting in B56 α phosphorylation and mitochondrial translocation. A mechanism whereby PKR may regulate Bcl2 dephosphorylation via B56 α has yet to be demonstrated and is currently under investigation. Still, it is tempting to speculate that PKR and the PP2A isoform that dephosphorylates Bcl2 are functionally linked through ceramide and B56 α.

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FIG. 9. Overexpression of exogenous B56 α promotes Bcl2 dephosphorylation in HL60 cells. HL60 parental cells or cells stably transfected with B56 α cDNA were radiolabeled with 32P in the absence or presence of 1 or 10 μM C2-ceramide for 3 h, and Bcl2 was immunoprecipitated. Phosphorylation was detected by autoradiography, and the identity of Bcl2 was confirmed by Western blot analysis as described previously (11).

FIG. 10. Overexpression of exogenous B56 α potentiates cell killing by ceramide. HL60 parental cells and B56 α transfectant clone 9 cells were treated for 24 h with increasing concentrations of ceramide. Cell viability was determined by trypan blue staining. Error bars represent the mean ± S.D. from three separate experiments.
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