De Novo Ceramide Regulates the Alternative Splicing of Caspase 9 and Bcl-x in A549 Lung Adenocarcinoma Cells

DEPENDENCE ON PROTEIN PHOSPHATASE-1

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Previous studies have demonstrated that several splice variants are derived from both the caspase 9 and Bcl-x genes in which the Bcl-x splice variant, Bcl-x(L) and the caspase 9 splice variant, caspase 9b, inhibit apoptosis in contrast to the pro-apoptotic splice variants, Bcl-x(s) and caspase 9. In a recent study, we showed that ceramide induces the dephosphorylation of SR proteins, a family of protein factors that regulate alternative splicing. In this study, the regulation of the alternative processing of pre-mRNA of both caspase 9 and Bcl-x(L) was examined in response to ceramide. Treatment of A549 lung adenocarcinoma cells with cell-permeable ceramide, D-e-C6 ceramide, down-regulated the levels of Bcl-x(L) and caspase 9b mRNA and immunoreactive protein with a concomitant increase in the mRNA and immunoreactive protein levels of Bcl-x(s) and caspase 9 in a dose- and time-dependent manner. Pretreatment with calyculin A (5 μM), an inhibitor of protein phosphatase-1 (PP1) and protein phosphatase 2A (PP2A) blocked ceramide-induced alternative splicing in contrast to okadaic acid (10 nM), a specific inhibitor of PP2A at these concentrations in cells, demonstrating a PP1-mediated mechanism. A role for endogenous ceramide in regulating the alternative splicing of caspase 9 and Bcl-x was demonstrated using the chemotherapeutic agent, gemcitabine. Treatment of A549 cells with gemcitabine (1 μM) increased ceramide levels 3-fold via the de novo sphingolipid pathway as determined by pulse labeling experiments and inhibition studies with myriocin (50 μM), a specific inhibitor of serine palmitoyltransferase (the first step in de novo synthesis of ceramide). Treatment of A549 cells with gemcitabine down-regulated the levels of Bcl-x(L) and caspase 9b mRNA with a concomitant increase in the mRNA levels of Bcl-x(s) and caspase 9. Again, inhibitors of ceramide synthesis blocked this effect. We also demonstrate that the change in the alternative splicing of caspase 9 and Bcl-x occurred prior to apoptosis following treatment with gemcitabine. Furthermore, doses of D-e-C6 ceramide that induce the alternative splicing of both caspase 9 and Bcl-x-sensitized A549 cells to daunorubicin. These data demonstrate a role for protein phosphatases 1 (PP1) and endogenous ceramide generated via the de novo pathway in regulating this mechanism. This is the first report on the dynamic regulation of RNA splicing of members of the Bcl-2 and caspase families in response to regulators of apoptosis.

Apoptosis (used here interchangeably with programmed cell death) is a mechanism (or group of mechanisms) by which cells execute endogenous programs of cell death, often in response to adverse external or internal signals or sources of injury (1–4). In the case of cancer, interference with this system of programmed cell death can lead to expansion of deleterious cells. Apoptosis is regulated by multiple factors through complex mechanisms (3, 4). It has become well established that many inducers of apoptosis activate caspases, and that the activation of these proteases is perhaps the point of irreversible commitment to the onset of apoptosis (5). Furthermore, the Bcl-2 family has also been widely implicated in regulating apoptotic machinery (6–9).

Several factors that regulate apoptosis have splice variants with an opposite/dominant negative function. The anti-apoptotic factor, Bcl-x(L), was the first shown to have a dominant negative splice variant, Bcl-x(s) (10). Dominant-negative splice variants of two members of the caspase family, caspase 2(s) and caspase 9b, have now also been described with both inhibiting apoptosis induced by most chemotherapeutic agents and extracellular agonists (11–13). Several splice variants of Bax have been described with the most recent, BaxΔ9, shown to block tumor necrosis factor α-induced apoptosis (14–19). Although recognized for metabolic and mitogenic pathways, the importance of alternative splicing in apoptosis and its mechanisms of regulation have been overlooked and largely unstudied.

In other lines of investigation, studies have led to the identification of ceramide as a potential inducer and mediator/regulator of apoptosis in response to tumor necrosis factor α and many chemotherapeutic agents (20–31). More recent studies have begun to relate the action of ceramide to Bcl-2 and death caspases (20–25, 32, 33).

While searching for direct targets of ceramide, a ceramide-activated protein phosphatase (CAPP) was identified. To date, two families of protein phosphatases, protein phosphatase-1 (PP1) and protein phosphatase 2A (PP2A), have been shown to...
be activated by ceramide in vitro (34–37). With the demonstration of PP1 as a ceramide-activated protein phosphatase, potential PP1 substrates and mechanisms regulated by PP1 became candidate targets for ceramide action.

SR proteins, a family of arginine/serine-rich domain containing proteins and specific PP1 substrates, are required for constitutive and alternative pre-mRNA processing (38–48). Endogenous ceramide has recently been found to modulate the phosphorylation status of SR proteins in a PP1-dependent manner (49). Several reports have also demonstrated a role for PP1 in regulating alternative splicing, and two splicingosomel targeting subunits for PP1 have been described (38, 48, 50, 51). Therefore, PP1 may play a role in regulating RNA processing in response to stimuli, in particular, it may define a pathway linking ceramide to the regulation of the alternative splicing of apoptosis regulators.

In this study, endogenous ceramides produced via the de novo sphingolipid pathway are shown to regulate the alternative pre-mRNA processing of caspase 9 and Bcl-x pre-mRNA. Furthermore, this novel and newly defined mechanism is shown to be mediated by protein phosphatase-1.

**MATERIALS AND METHODS**

**Cell Culture—**A549 adenocarcinoma cells were grown in a 50% RPMI 1640 (Invitrogen) and 50% Dulbecco’s modified Eagle’s medium (In-vitrogen) mixture supplemented with 1-glutamine, 10% (v/v) fetal bovine serum (Sigma), 200 units/ml penicillin G sodium, and 200 μg/ml streptomycin sulfate. Cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37°C). For treatments with D-e-C6 ceramide, A549 cells were plated at 4 x 104 cells/55-mm plate in RPMI 1640 and Dulbecco’s modified Eagle’s medium supplemented with 2% (v/v) fetal bovine serum.

**RNA Extraction—**Total RNA was extracted using Trizol reagent (Invitrogen). The sample was then separated into an upper aqueous phase and a lower organic phase by centrifugation at 12,000 × g for 15 min at 4°C. The aqueous phase was transferred to a new tube and the RNA was precipitated by the addition of 1 volume of isopropyl alcohol. The precipitant was incubated for 10 min at room temperature, and RNA was collected by centrifugation at 12,000 × g for 15 min at 4°C. The pellet was washed with 1 ml of 75% ice-cold ethanol, and the RNA was resuspended in nuclease-free water for storage at −80°C.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—**For both Bcl-x and caspase 9 analysis, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) as the primering agent. After 1 h incubation at 43.5°C, the reactions were stopped by 70°C heating for 15 min. Template RNA was then removed using RNase H (Invitrogen).

For evaluating Bcl-x splice variant expression, an upstream 5’ primer to Bcl-x (5′-GAGCAGGAGGCAGGTCTTTTGAA-3′) and a 3′ primer (3′-TGGAGGCTAGTGAGGTGT5′) were used. Using these primers, 10% of the reverse transcriptase reaction was amplified for 35 cycles (94°C, 30s; 58°C, 30s; 72°C, 1 min) using Platinum Taq DNA polymerase (Invitrogen).

For evaluating caspase 9 splice variant expression, an upstream 5’ primer to caspase 9 (5′-GCTCTTCCTTTGTTCATCTCC-3′) and a 3′ primer (5′-CATCTGGCTCCGGGGTCTGTC-3′) were used. Using these primers, 10% of the reverse transcriptase reaction was amplified for 35 cycles (94°C, 30s; 58°C, 30s; 72°C, 1 min) using Platinum Taq DNA polymerase (Invitrogen).

**Construction and Labeling of Riboprobes—**The caspase 9 and Bcl-x riboprobe templates were constructed by cloning the RT-PCR fragments for caspase 9 (724 bp) and caspase 9b (274 bp) into pCMV-Blunt II TOPo vector. Constructs were verified for orientation and the correct sequence by DNA sequencing. To produce caspase 9 cRNA, the riboprobe construct for caspase 9 was restriction digested with NciI, and caspase 9 cRNA (32P-labeled) was produced using the Bcl Pharmingen in vitro transcription system and SP6 RNA polymerase (Sigma). To produce cRNA for caspase 9b, the riboprobe construct for caspase 9b was restriction digested with BamHI, and caspase 9b cRNA (32P-labeled) was produced using the BD Pharmingen in vitro transcription system. The Bcl-x(L/s) riboprobe template was part of the hApo2 multitemplate set from BD Pharmingen and labeled following the standard protocol.

**Ribonuclease Protection Assays—**Total RNA (5 μg) from A549 cells was hybridized to 500,000 cpm of 32P-labeled cRNA probe using the BD Pharmingen ribonuclease protection assay system. RNase-protected fragments were produced following the manufacturer’s protocol. Protected RNA fragments were resolved on 5% PAGE-t at urea gels (Bio-Rad), dried at 80°C for 1 h, and autoradiograms produced using BioMax film (Kodak).

**Protein Extraction—**Total protein was extracted by direct lysis with Laemmli buffer. Cells were lysed with 0.1 ml of 2 × Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.04% bromophenol blue, and 250 mM β-mercaptoethanol) after resuspension in 0.1 ml of ice-cold phosphate-buffered saline (PBS). Samples were boiled for 10 min and either examined directly by SDS-polyacrylamide gel electrophoresis or stored at −20°C.

**Western Immunoblotting—**Total protein lysate (20 μg) was subjected to 6% (PARP), 12% (caspase 9/9b), or 15% (Bcl-xL/s) SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and blocked in 5% milk, 1 × PBS-T (M-PBS-T) for 2 h. The membrane was probed with anti-PARP (Santa Cruz), anti-Bcl-x IgG (Santa Cruz (P19)), or anti-caspase 9 IgG (Stressgen) for 2 h in M-PBS-T followed by 3 washes with PBS-T. The membrane was then incubated with a secondary antibody of horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody for 45 min followed by 3 washes with PBS-T. Immunoblots were developed using Amersham ECL reagents and Bio-Max film.

**Quantification of Ceramide Levels: Pulse Labeling with [3H]palmitic Acid—**Two × 106 A549 cells were incubated with 1 μCi/ml [3H]palmitic acid (16.7 μCi) with simultaneous addition of gemcitabine. After 24 h, lipids were extracted from the radiolabeled cells using the Bligh-Dyer method as described (52). Ceramide levels were measured following TLC analysis and normalized to total lipid phosphate as described (53, 54).

**MTT Assay—**1.5 × 105 A549 cells were plated into each well of a 96-well plate in a 50-μl volume. After 24 h at standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37°C), the cells were treated with the appropriate concentration of D-e-C6 ceramide or gemcitabine in a 50-μl volume and returned to the incubator. After the appropriate time, 25 μl of MTT solution (5 mg/ml) was added and cells were again incubated under standard conditions for 5 h. Cells were then lysed and solubilized by the addition of 100 μl of lysis solution (20% SDS (w/v), 50% dimethyl formamide (v/v), and 0.8% acetic acid). The plate was read at 595 nm.

**Statistical Analysis—**Tests were done in triplicate on at least two separate occasions. The standard deviations and standard error of mean were determined after quantitating a change in the ratio of one alternative splice variant versus another following treatment as compared with untreated and sham control samples. It was determined that a two-way ANOVA at p < 0.05 to be significant. All statistical tests were done with StatView software (SII Corp.) and Microsoft EXCEL.

**RESULTS**

**Exogenous Ceramide Regulates the Alternative Splicing of Bcl-x and Caspase 9 Pre-mRNA—**We have previously reported that SR proteins, a family of factors that regulate alternative splicing, are dephosphorylated in a time- and dose-dependent manner in response to ceramide (49). Since many apoptosis regulating factors have alternative splice variants with antagonistic function, ceramide was examined for effects on the pre-mRNA processing of Bcl-x, caspase 9, Bax, and caspase 2. Using an RT-PCR-based assay (Fig. 1, A and B), it was found that overnight treatment of A549 lung adenocarcinoma cells with 20 μM D-e-C6 ceramide (sub-Ic50, dose for a 24-h period in A549 cells (IC50 = 37 μM) resulted in altering the ratio of the splice variants of Bcl-x and caspase 9 (Fig. 2, A and B), but not Bax or caspase 2 (data not shown). In the case of Bcl-x, there was a decrease in the ratio of Bcl-x(L)/Bcl-x(s) from 9.1 to 4.3 (Fig. 2A), and in the case of caspase 9, there was an increase in the ratio of caspase 9/caspase 9b from 6.5 to 22.7 (Fig. 2B).

Since the RT-PCR-based assay is semiquantitative and as-
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A

Bcl-x Gene Structure

Exon 1

Intron 1

Exon 2

Bcl-x(L) PCR Product (466 bp)

Bcl-x(L) RPA Protected Fragments (364 bp)

Bcl-x(s) PCR Product (711 bp)

Bcl-x(s) RPA Protected Fragments (314 bp)

Exon 3

The Bcl-x(s)-specific 5’ Splice Site

Intron 2

The Bcl-x(L)-specific 5’ Splice Site

Exon 4

The Bcl-x(s)-specific 5’ Splice Site

Exon 5

The Bcl-x(L)-specific 5’ Splice Site

Exon 6

Exon 7

B

Caspase 9 Gene Structure

Exon 2

Intron 2

Intron 6

Exon 3

3,4,5,6 Cassette

Exon 4

Exon 5

Caspase 9 RT-PCR Product (741 bp)

Caspase 9 RPA Protected Fragment (421 bp)

Caspase 9b RT-PCR Product (274 bp)

Caspase 9b RPA Protected Fragment (274 bp)

A. The schematic depicts the gene structure of Bcl-x, which is composed of three exons and two introns. The Bcl-x(L)- and Bcl-x(s)-specific 5’ splice sites are designated. Arrows depict the location of PCR primers used in the RT-PCR assay. The figure also depicts the PCR fragments obtained from the RT-PCR assay and the protected RPA fragments obtained in assaying Bcl-x(L) and Bcl-x(s) mRNA levels. B. The schematic depicts the gene structure of caspase 9 with the exon 3, 4, 5, and 6 cassettes specific for caspase 9 mRNA. Arrows depict the location of PCR primers used in the RT-PCR assay. The figure also depicts the PCR fragments obtained from the RT-PCR assay and the protected RPA fragments obtained in assaying caspase 9 and caspase 9b mRNA levels.

Figs. 1A and 1B depict the location of PCR primers used in the RT-PCR assay. The schematic depicts the gene structure of Bcl-x, which is composed of three exons and two introns. The Bcl-x(L)- and Bcl-x(s)-specific 5’ splice sites are designated. Arrows depict the location of PCR primers used in the RT-PCR assay. The figure also depicts the PCR fragments obtained from the RT-PCR assay and the protected RPA fragments obtained in assaying Bcl-x(L) and Bcl-x(s) mRNA levels.

The effects of mRNA levels translated to the protein level as ceramide treatment decreased the immunoreactive protein levels of Bcl-x(L) by 57% with a concomitant 2.5-fold increase in the immunoreactive protein levels of Bcl-x(s) (Fig. 3A). Similarly at the protein level, ceramide induced an increase in the immunoreactive protein levels of caspase 9 and a concomitant decrease in the immunoreactive protein levels of caspase 9b (Fig. 3B). Thus, since ceramide increases one splice variant followed by a decrease in the other splice variant, these data demonstrate that ceramide affects the alternative splicing of caspase 9 and Bcl-x in a manner that promotes apoptosis.

The effects of ceramide on the pre-mRNA processing of Bcl-x and caspase 9 were time- and dose-dependent. For caspase 9, an increase in the caspase 9/caspase 9b ratio was noticeable by 12 h, and 5 μM D-e-C6 ceramide induced profound effects on the alternative splicing of caspase 9, increasing the ratio of caspase 9/caspase 9b to 14.6 with a maximal increase of 22.7 at 20 μM after 24 h (Fig. 4, A and B). In contrast, ceramide did not affect the alternative splicing of Bcl-x until after 16 h of ceramide treatment with maximal effects at 36 h (Fig. 4C). Similar to caspase 9, at least 5 μM D-e-C6 ceramide was necessary to induce a significant decrease in the ratio of Bcl-x(L)/Bcl-x(s) (Fig. 4D). Therefore, the effect of ceramide treatment of the alternative splicing of caspase 9 and Bcl-x is dose- and time-dependent.

Ceramide-induced Alternative Splicing Is Inhibited by Calyculin A, but Not Okadaic Acid—Previously, the dephosphorylation of SR proteins in response to the generation of endogenous ceramide was found to be dependent on PP1, a ceramide-activated protein phosphatase (49). To establish whether a ceramide-activated protein phosphatase plays a role in regulating the alternative splicing of caspase 9 and Bcl-x, we pre-treated A549 cells for 2 h with 5 nM calyculin A, an inhibitor of both PP1 and PP2A-type protein phosphatases. Calyculin A completely blocked the ceramide effects on caspase 9 and Bcl-x alternative splicing (Fig. 5, A and B). To establish whether PP1 or PP2A was the ceramide-responsive protein phosphatase regulating caspase 9 and Bcl-x alternative splicing, A549 cells were pretreated for 2 h with 10 nM okadaic acid, a selective PP2A inhibitor at this dose in cells (49, 55, 56). Pretreatment with okadaic acid had no effect on either caspase 9 or Bcl-x alternative splicing (Fig. 5, A and B). Calyculin A, but not okadaic acid also inhibited the effect of ceramide on the immunoreactive levels of caspase 9 and Bcl-x. Taken together, these results suggest that PP1 mediates the effects of ceramide on the alternative splicing of both caspase 9 and Bcl-x.

Ceramide-induced Alternative Splicing Is Inhibited by Fumonisin B1, an Inhibitor of CoA-dependent Ceramide Synthase—Exogenous ceramide treatment has been demonstrated to increase endogenous ceramide via deacylation/reacylation in A549 cells. A key enzyme in this pathway is CoA-dependent ceramide synthase, which is composed of three exons and two introns. The Bcl-x(L)- and Bcl-x(s)-specific 5’ splice sites are designated. Arrows depict the location of PCR primers used in the RT-PCR assay. The figure also depicts the PCR fragments obtained from the RT-PCR assay and the protected RPA fragments obtained in assaying Bcl-x(L) and Bcl-x(s) mRNA levels.

Endogenous Ceramide Generated by the de Novo Sphingolipid Pathway Induces the Alternative Splicing of Caspase 9 and Bcl-x—Since CoA-dependent ceramide synthase is an enzyme in the de novo biosynthetic pathway of ceramide, we established a model in A549 cells for generation of de novo ceramide in response to extracellular agents. The de novo synthesis of ceramide was assessed directly by pulse labeling A549 cells with [3H]palmitic acid. Treatment of A549 cells with the chemotherapeutic drug, gemcitabine (1 μM), for 24 h induced a 3.1-fold increase in [3H]ceramide (Fig. 6A). Pretreatment with

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myriocin, a specific inhibitor of serine palmitoyltransferase (the first enzyme in sphingolipid biosynthesis), blocked the increase in \[^3H\]ceramide following 24 h of gemcitabine exposure (Fig. 6A). Furthermore, pretreatment of A549 cells with myriocin (50 nM), reduced the basal levels of \[^3H\]ceramide by 62% (Fig. 6A). Thus, the increase in ceramide levels in response to gemcitabine occurs via the de novo sphingolipid pathway in A549 cells.

To establish that ceramide generated via the de novo sphingolipid pathway will induce a change in the alternative splicing of caspase 9 and Bcl-x, we again treated A549 cells with gemcitabine for 24 h. Using the RT-PCR based assay, we demonstrated that treatment with gemcitabine (1 μM) induced an increase in the ratio of caspase 9 mRNA/caspase 9b mRNA from 5.1 to 19.2. Furthermore, the ratio of Bcl-x(L) to Bcl-x(s) was decreased significantly from 8.7 to 3.8 (Fig. 6B and C). To demonstrate that this mechanism was dependent on the generation of ceramide via the de novo sphingolipid pathway, A549 cells were pretreated with myriocin (50 nM). Pretreatment with myriocin blocked gemcitabine-induced alternative splicing (Fig. 7A and B). Thus, the alternative splicing of caspase 9 and Bcl-x are regulated by the generation of endogenous ceramide via the de novo sphingolipid pathway.

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myriocin, a specific inhibitor of serine palmitoyltransferase (the first enzyme in sphingolipid biosynthesis), blocked the increase in \[^3H\]ceramide following 24 h of gemcitabine exposure (Fig. 6A). Furthermore, pretreatment of A549 cells with myriocin (50 nM), reduced the basal levels of \[^3H\]ceramide by 62% (Fig. 6A). Thus, the increase in ceramide levels in response to gemcitabine occurs via the de novo sphingolipid pathway in A549 cells.

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Ceramide-induced Alternative Splicing Occurs Prior to Apoptotic Cell Death and Sensitizes A549 Cells to Daunorubicin—To determine whether the increase in pro-apoptotic caspase 9 and Bcl-x(s) and decrease in anti-apoptotic caspase 9b and Bcl-x(L) occurs prior to apoptosis, we determined the time course of apoptosis following treatment with gemcitabine. Treatment of A549 cells for 24 h with gemcitabine (1 μM) did not induce significant cell death as measured by MTT assay.
and there was no observed cleavage of PARP following these treatments (Fig. 8). Significant cell death was not observed until after 72 h of treatment (58%) by MTT assay, and PARP cleavage was also not observed until after 72 h (Fig. 8). Thus, the change in the alternative splicing of caspase 9 and Bcl-x occurs prior to apoptosis induced by gemcitabine in A549 cells.

To determine whether ceramide-induced alternative splicing may play a role in sensitization of cells to chemotherapy, we treated A549 cells with various doses of daunorubicin in the presence of either vehicle, 1, 5, 10, or 20 μM D-e-C₆ ceramide. Treatment of A549 cells with 5, 10, or 20 μM D-e-C₆ ceramide significantly lowered the IC₅₀ of daunorubicin from 3.7 μM to 0.65, 0.425, and 0.425 μM, respectively (Table I). Treatment of A549 cells with a dose of D-e-C₆ ceramide (1 μM) that has no effect on the alternative splicing of caspase 9 and Bcl-x, had no effect on the IC₅₀ of daunorubicin (Table I). Thus, ceramide-
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**FIG. 6.** The effects of gemcitabine treatment on the levels of ceramide and the alternative splicing of Bcl-x and caspase 9. A, the effect of gemcitabine on the levels of [3H]ceramide following pulse labeling with [3H]palmitate. A549 cells were treated for 24 h with or without 50 nM myriocin followed by treatment with 1 μM gemcitabine. Total lipids were extracted using the Bligh-Dyer method, and the extracted lipids were base hydrolyzed, subjected to thin layer chromatography (TLC), and autoradiographed. The results are presented as arbitrary densitometry units of labeled ceramide. Samples were normalized to nanomoles of total lipid phosphatase prior to TLC. Data are representative of three separate determinations on two separate occasions. B, the effect of gemcitabine on the alternative splicing of caspase 9. A549 cells were treated for 24 h with 1 μM gemcitabine. Total RNA was extracted and analyzed by RT-PCR for gemcitabine-induced alternative splicing. Data are representative of three separate determinations on two separate occasions. C, the effect of gemcitabine on the alternative splicing of Bcl-x. A549 cells were treated for 24 h with 1 μM gemcitabine. Total RNA was extracted and analyzed by RT-PCR for gemcitabine-induced alternative splicing. Data are representative of three separate determinations on two separate occasions.

Induced alternative splicing of caspase 9 and Bcl-x correlates with sensitization of A549 cells to daunorubicin.

**DISCUSSION**

Recently, we reported that ceramide, a known inducer of apoptosis, regulated the phosphorylation state of SR proteins, a family of RNA transactivating factors that regulate alternative splicing (49). Based on these previous findings and on the observations that several protein factors which regulate apoptosis have splice variants with a dominant/negative function, we examined whether the processing of Bcl-x, caspase 9, Bax, and caspase 2 pre-mRNA was affected by ceramide. The results from this study demonstrate that ceramide induces, via alternative splicing, the levels of pro-apoptotic splice variants Bcl-x(s) and caspase 9, with a concomitant loss in the anti-apoptotic splice variants, Bcl-x(L) and caspase 9b. This newly defined and novel mechanism was also demonstrated to be dependent on both PP1 activation and endogenous ceramide. These observations are important for several reasons. First, this mechanism of ceramide-induced alternative splicing defines a novel mechanism of regulating the gene expression of pro-apoptotic factors in response to extracellular agents. Second, a direct and specific mechanism mediated by a ceramide-activated protein phosphatase and endogenous ceramide generated via the de novo sphingolipid pathway has been defined. Third, a new mechanism involved in the initiation stage of apoptosis and the sensitization of cells to chemotherapy has been implicated.

Previous studies on agonist-induced alternative splicing have mainly focused on mitogenic pathways activated by hormones and growth factors such as insulin and insulin-like growth factor 1 which have been shown to regulate the alternative splicing of the insulin receptor, protein kinase Cβ, and protein-tyrosine phosphatase-1 (45, 59–66). To our knowledge, the results in this study describe, for the first time, a novel mechanism by which apoptotic stimuli can affect gene expression through alternative splicing. In this case, ceramide, a mediator of apoptosis, reduced the expression of the cell survival factors caspase 9b and Bcl-x(L) and increased the expression of the pro-apoptotic factors Bcl-x(s) and caspase 9. This was demonstrated by a semi-quantitative assay based on RT-PCR which demonstrated a change in the ratio of caspase 9 to caspase 9b and in the ratio of Bcl-x(L) to Bcl-x(s). To verify that both splice variants were being affected over the time periods presented, we used a ribonuclease protection assay that was highly quantitative. Using this assay, Bcl-x(L) was shown to decrease with ceramide treatment (following the published half-life of ~14 h for Bcl-x(L) mRNA) as well as caspase 9b while caspase 9 and Bcl-x(s) were both demonstrated to in-
increase significantly (67, 68). Since both splice variants are affected in response to ceramide, transcriptional effects are an unlikely mechanism of action as both splice variants would be affected in the same direction.

The mechanisms that regulate the gene expression of either caspase 9 or Bcl-x are of direct relevance to the apoptotic mechanism as both caspase 9 and Bcl-x have been demonstrated to be important mediators of apoptosis. More specifically, the differential regulation of apoptosis by isoforms of both Bcl-x and caspase 9 generated via alternative splicing is of importance as overexpression of either caspase 9 or Bcl-x(s) induces apoptosis, whereas overexpression of caspase 9b and Bcl-x(L) has been shown to inhibit apoptosis in response to Fas, tumor necrosis factor α, Bax, TRAIL, UV radiation, and Bik (11, 12, 75–81). Moreover, recent reports have demonstrated a role for the alternative splicing of Bcl-x in chemotherapy sensitivity and induction of apoptosis. These studies reported that exposure of A549 cells (24 h) to an oligonucleotide that specifically interacted and blocked the 5′ splice site for Bcl-x(L) within exon 2, induced down-regulation of Bcl-x(L) with a concomitant increase in Bcl-x(s) (69, 70). Simultaneously, the IC50 of daunorubicin for A549 cells was decreased by 80% (69, 70). Furthermore, chronic exposure (>24 h) to the oligonucleotide-induced apoptosis (71). Thus, by affecting the alternative splicing of Bcl-x pre-mRNA, cells become more sensitive to chemotherapy and undergo apoptosis. This mechanism would promote apoptosis by increasing cell death components with the simultaneous attenuation of cell survival factors (10–12, 72–74). Thus, the current studies define a novel mechanism of regulating the alternative splicing of regulators of apoptosis in response to ceramide.

Of interest, Bcl-x(L) mRNA still predominated after ceramide treatment. Thus the question is raised as to whether enough Bcl-x(s) is expressed to overcome normal Bcl-x(L) expression in cells. Thompson and co-workers (10) demonstrated that only 1 molecule of Bcl-x(s) per 4 molecules of Bcl-x(L) was necessary to overcome the Bcl-x(L) survival mechanism. Thus, in this study, ceramide lowered the ratio of Bcl-x(L)/Bcl-x(s) mRNA from 9.3 to 4.1. This was reflected at the protein level by a decrease in Bcl-x(L) and an increase in Bcl-x(s) immunoreactive protein. If the mRNA ratio reflects the protein ratio of Bcl-x(L)/Bcl-x(s), this new ratio of Bcl-x(L)/Bcl-x(s) would promote apoptosis. A similar case was demonstrated for activation of caspase 9. Alnemri and co-workers (12) showed that only 1 molecule of caspase 9b to 4 protein molecules of caspase 9 completely blocked the activation of caspase 9 in vitro. In the current study, ceramide enhanced the ratio of caspase 9/caspase 9b mRNA from 5.1 (95% inactive caspase 9 in vitro) to almost 20:1 (fully active caspase 9 in cells). Interestingly, the protein levels of caspase 9b were higher than caspase 9 which did not correspond to the mRNA ratio of caspase 9 to caspase 9b in A549 cells as determined by RT-PCR. On the other hand, the protein levels of caspase 9 and 9b were reflected closely by the RPA assays which are more quantitative. This is explained by the high GC-rich content of the caspase 9b mRNA/cDNA which lowers its extension rate compared with caspase 9 mRNA/cDNA during PCR by 80% (data not shown). Since a ratio for caspase 9/caspase 9b mRNA cannot be established using the presented RPA data, an additional role for ceramide regulation of either translational efficiency of the caspase 9b mRNA or different protein stabilities between caspase 9 and caspase 9b cannot be completely ruled out.

Mechanistically, ceramide-induced alternative splicing was dependent on the activation of PP1. This conclusion was based on the use of the potent inhibitors of serine/threonine-protein phosphatases, okadaic acid, and calyculin A. In this study, we demonstrate that the PP1 and PP2A inhibitor, calyculin A, completely blocked ceramide-induced alternative splicing of
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Bcl-x and caspase 9. On the other hand, okadaic acid, a specific inhibitor of PP2A at the concentrations used, had no effect on ceramide-induced alternative splicing of Bcl-x and caspase 9. This therefore infers that the mechanism is dependent of PP1 activation. This conclusion is supported by several previous reports. First, natural ceramides have been shown to activate PP1 in a stereospecific manner in vitro (37). Second, activation of PP1 in cells has been demonstrated to occur in response to the generation of endogenous ceramide via CoA-dependent ceramide synthase and via the de novo sphingolipid pathway. Third, the dephosphorylation of a family of RNA splice factors, SR proteins, has been demonstrated to require the activation of PP1 (49). The role of SR proteins in ceramide-induced alternative splicing may play a role in producing a pro-apoptotic phenotype with enhanced sensitization of cells to apoptotic stimuli. Studies are under way to investigate this possibility as well as the possible role in chemotherapeutic resistance.

In conclusion, these results demonstrate a novel mechanism in which endogenous ceramide and PP1 regulate the alternative splicing of specific apoptotic factors, caspase 9 and Bcl-x. This mechanism may have direct relevance to the action of chemotherapeutic agents that function to induce intracellular levels of ceramide, such as etoposide, Ara-C, Taxol, gemcitabine, and daunorubicin (Fig. 9) (29, 54, 75–81).

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