Retinoblastoma gene product as a downstream target for a ceramide-dependent pathway of growth arrest

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ABSTRACT Ceramide, a lipid mediator, has been most closely associated with antiproliferative activities. In this study, we examine the mechanism by which ceramide induces growth suppression and the role of the retinoblastoma gene product (Rb) in this process. Withdrawal of serum from the serum-dependent MOLT-4 cells resulted in significant dephosphorylation of Rb, correlating with the induction of G₀/G₁ cell cycle arrest. Serum withdrawal resulted in marked elevation in the levels of endogenous ceramide (3-fold at 24 h and 8-fold at 96 h) with little changes in the endogenous levels of sphingosine. The addition of exogenous C₆-ceramide resulted in a concentration- and time-dependent dephosphorylation of Rb. Exogenous ceramide was active at levels comparable to endogenous levels achieved with serum withdrawal. Peak activity of exogenous ceramide (at 6 h) correlated with the uptake of C₆-ceramide by MOLT-4 cells. Next, a number of studies were conducted to determine whether Rb plays a role in ceramide-induced growth suppression. (i) C₆-Ceramide was poorly active in growth suppression of retinoblastoma cells that lack Rb. (ii) Mink lung epithelial cells in which Rb had been sequestered by overexpression of large tumor antigen were resistant to the action of ceramide compared to cells transfected with large tumor antigen mutated in the Rb-binding pocket. (iii) Overexpression of the E1A adenoviral protein, which binds and sequesters Rb, resulted in protection from growth suppression and cell cycle arrest induced by ceramide. Thus, these studies demonstrate that Rb is a downstream target for ceramide and may function in a growth suppressor pathway resulting in cell cycle arrest.

In contrast to the advanced molecular and biochemical understanding of mitogenic pathways, insight into growth suppressor pathways is only now being developed. The sphingomyelin/ceramide pathway has recently emerged as a candidate regulatory signaling mechanism mediating antiproliferative activity and apoptosis (1, 2). In this proposed signal transduction pathway, the action of a number of extracellular agents such as 1 α ,25-dihydroxyvitamin D₃ (3) and the cytokines tumor necrosis factor α (4–6), γ -interferon (4), and interleukin 1 (7, 8) results in activation of a neutral sphingomyelinase, causing hydrolysis of membrane sphingomyelin and generation of ceramide. In turn, ceramide has been shown to mediate a number of biochemical activities such as inhibition of expression of the *c-myc* protooncogene (9), induction of cyclooxygenase (7), enhancement of phosphorylation of the epidermal growth factor receptor (10), and modulation of activity of protein kinases and phosphatases (11–13). At a cellular level, ceramide has been shown to exert antiproliferative effects. A significant component of the growth suppression induced by ceramide appears to be a result of the induction of apoptosis. Ceramide, but not closely related analogs, induces programmed cell death in a number of cell lines (1, 2) mimicking

the actions of tumor necrosis factor α , ionizing radiation (14), and the chemotherapeutic agent 1- β -D-arabinofuranosylcytosine (15) that have been shown to stimulate ceramide generation. Whether ceramide activates other growth suppressive effects (such as cell cycle arrest) has not been examined.

Recently, ceramide has also been shown to induce a G₀/G₁ arrest in cell cycle progression, presumably mediating the cell cycle arrest observed in response to withdrawal of essential serum factors (42). However, the mechanism by which ceramide impacts on the cell cycle has not been defined. In this study, we demonstrate that ceramide induces dephosphorylation of the retinoblastoma gene product (Rb) since Rb has been implicated as a key inhibitor of cell cycle progression (16–18). The early and potent effects of Rb dephosphorylation that are specific to ceramide (and not other lipids) support a role for endogenous ceramide in mediating the effects of serum withdrawal. Through the use of cell lines that lack Rb or in which Rb is sequestered/bound, evidence is provided for a necessary role for Rb in mediating the growth suppressive and cell cycle effects of ceramide. The implications of these studies on defining a sphingolipid-dependent pathway of cell cycle regulation are discussed.

EXPERIMENTAL PROCEDURES

Ceramide Synthesis. D-erythro-C₆-Ceramide was synthesized from (2S,3R)-sphingosine prepared in stereospecific synthesis using L-serine. DL-erythro-Dihydro-C₆-ceramide was synthesized from DL-erythro-dihydrosphingosine (Sigma D7033) as described (19).

Rb Phosphorylation. Cells (10 ml of 2 $\times$ 10⁵ cells per ml) were collected by centrifugation through 10% (vol/vol) Percoll (Pharmacia) in buffer A (25 mM Hepes, pH 7.4/90 mM NaCl/1 mM EDTA/1 mM EGTA/1 mM sodium orthovanadate/10 mM NaF). Proteins were collected and Western blot analyses for Rb were performed essentially as described (20) with 40 μ g of protein per well. Protein concentration was determined in 5 μ l of cell suspension by using the Pierce protein determination kit.

Ceramide Measurements. MOLT-4 cells, seeded at 5 $\times$ 10⁵ cells per ml, were placed in serum-free medium for the indicated times. Cells were harvested and lipids were extracted by the Bligh and Dyer method (21); lipids were dried and resuspended in 100 μ l of chloroform. Duplicate aliquots of 25 μ l were set aside for phosphate measurements (22) and 25 μ l was utilized in the ceramide assay using *Escherichia coli* diacylglycerol kinase (23, 24). Ceramide was quantitated by using external standards and was normalized to phosphate.

Sphingosine Measurements. Sphingosine mass measurements were performed essentially by the method of Merrill et

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Abbreviations: Rb, retinoblastoma gene product; TAg, large tumor antigen; FBS, fetal bovine serum.

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al. (25) after *ortho*-phthaldehyde derivatization and HPLC analysis on a Nova-Pack C₁₈ reverse-phase column and by using C₂₀-sphingosine as an internal standard.

C₆-Ceramide Metabolism. Ceramide metabolism was evaluated by utilizing ceramide labeled in the sphingosine backbone, *N*-[3-³H]hexanoylsphingosine (New England Nuclear). MOLT-4 cells, seeded at 2×10^5 cells per ml, were preincubated in 10 ml of 2% (vol/vol) fetal bovine serum (FBS) RPMI 1640 medium for 1–2 h before treatment with 10 μ M C₆-ceramide (3 μ Ci; 1 Ci = 37 GBq). At the indicated times, cells were harvested and 2 ml of chloroform/methanol, 1:1 (vol/vol), and 100 μ l of water were added to the cell pellets. After overnight extraction, mixtures were centrifuged and supernatants were transferred into new tubes. The solvents were removed by evaporation and lipids were resuspended into 100 μ l of chloroform/methanol. The lipids were applied to TLC plates and separated by using one of three solvent systems: system A, 1-butanol/acetic acid/water, 3:1:1 (vol/vol) (to separate sphingosine metabolites); system B, chloroform/methanol/28% (wt/vol) ammonium hydroxide, 4:1:0.1 (vol/vol) (to separate ceramides); system C, chloroform/methanol/glacial acetic acid/water, 50:30:8:4 (vol/vol) (to separate phospholipids). C₆-Ceramide and sphingosine were applied as standards to identify the C₆-ceramide spot and any metabolism to sphingosine. After chromatography, plates were dried, developed in I₂, sprayed with EN³HANCE spray (DuPont), and subjected to autoradiography for 1–6 days. Spots containing radioactive compounds were scraped and radioactivity was measured by using a 1209 Rackbeta scintillation counter.

Retroviral Expression of E1A. The E1A-expressing MOLT-4 (MOLT-4-E1A) was generated by using retroviral-mediated gene transfer. The plasmid puc118E1A was cloned into the *Eco*RI-*Bam*HI site of the retrovirus vector LXSN (26). Retrovirus was generated by transient cotransfection of COS cells with this construct, which contains the retroviral packaging signal, and pSV-(Ψ^-)A-MLV (27), which lacks the packaging signal. LXSN was used in a parallel cotransfection as control to generate the cell line, MOLT-4-LXSN. Supernatants from each of the cotransfections were used to infect growing MOLT-4 cells. Two days after infection, cells were selected with G418 at 400 μ g/ml. Whole cell extracts were prepared from both cell lines to verify E1A expression by Western blot analysis with E1A monoclonal antibody (PharMingen). For cell cycle analysis, 2×10^6 MOLT-4-LXSN or MOLT-4-E1A cells were harvested, resuspended in 1 ml of PBS, and fixed in absolute ethanol at -20°C . Cells were stored at -20°C until the day of analysis, at which time, the cells were pelleted, and the fixation solution was removed. Cells were resuspended in 1 ml of PBS and incubated in the presence of DNase-free RNase (20 μ g/ml) for 30 min at 37°C . Cells were then stained with propidium iodide (100 μ g/ml) for 5–10 min. Flow cytometric analysis was performed with an argon laser at 488 nm and a Becton Dickinson FACStar^{Plus} flow cytometer.

RESULTS

MOLT-4 cells are a useful model for the study of serum factor-dependent growth. In this cell line, withdrawal of serum results in ceramide accumulation, and ceramide induces a G₀/G₁ block in cell cycle progression (42). Because Rb is a critical regulator of G₁/S progression, we attempted to determine the regulation of Rb (by phosphorylation) and the role of ceramide in this process. Initial experiments were directed at determining whether Rb participates in the process of serum-withdrawal-induced cell cycle arrest. Therefore, the effects of serum deprivation on the state of Rb phosphorylation in MOLT-4 cells were investigated. Rb dephosphorylation was evident at 24 h after serum deprivation, became increasingly more pronounced at 48 and 72 h, and was almost complete by 96 h (Fig. 1A). These results show that Rb is regulated in this process of growth arrest.

FIG. 1. Effect of serum deprivation on Rb dephosphorylation and endogenous sphingolipid levels. (A) Rb dephosphorylation with serum deprivation. MOLT-4 cells grown in 10% FBS were washed with serum-free RPMI 1640 medium, resuspended in serum-free medium, and harvested at the indicated times. The status of Rb phosphorylation was determined by Western blot analysis since hyperphosphorylated Rb migrates more slowly than the hypophosphorylated forms on SDS/polyacrylamide gel electrophoresis (28). (B) The effect of serum deprivation on endogenous ceramide and sphingosine levels. The mass levels of ceramide (○) and sphingosine (■) were normalized to total phospholipids. Similar results were obtained when mass levels were normalized against cell numbers.

Although the dephosphorylated form of Rb is correlated with activation, the signaling mechanisms resulting in Rb dephosphorylation remain poorly defined. *D-erythro*-Sphingosine, a sphingolipid breakdown product, induces rapid and potent Rb dephosphorylation (20). So far, however, no physiologic role for sphingosine in regulating Rb activity has been described because of the lack of documented changes in intracellular sphingosine in the setting of Rb dephosphorylation. We wondered whether ceramide or sphingosine play a role in serum-withdrawal-induced Rb dephosphorylation. Because of the close metabolic relationship between sphingosine and ceramide (product-precursor), it became important to determine the effects of serum deprivation on endogenous sphingosine and ceramide levels. Sphingosine mass levels were measured from total lipid extracts 10, 24, 48, and 72 h after serum deprivation of MOLT-4 cells. As is evident in Fig. 1B, sphingosine levels did not change significantly with prolonged serum deprivation, while a marked increase in endogenous ceramide levels was observed that started at 24 h and continued to increase even at 96 h. Importantly, the elevation in ceramide levels correlated temporally with Rb dephosphorylation observed with serum deprivation (Fig. 1).

Since ceramide, rather than sphingosine, appears to be the physiologically relevant sphingolipid breakdown product during serum deprivation, it became necessary to determine whether ceramide, on its own, could induce Rb dephosphorylation. Rb phosphorylation status was assessed in MOLT-4 cells treated for 4 h with increasing concentrations of the short-chain synthetic ceramide C₆-ceramide. There was a dose-dependent induction of Rb dephosphorylation (Fig. 2A). Hypophosphorylated (fast-migrating) Rb species appeared at 10 μ M C₆-ceramide and predominated at 20 μ M. These concentrations of exogenous ceramide achieve cellular levels comparable to the endogenous levels after serum withdrawal. Rb dephosphorylation by C₆-ceramide was also time-dependent: at 10 μ M C₆-ceramide, hypophosphorylated Rb species appeared after 4 h of treatment and became more predominant at 6 and 8 h (Fig. 2B). Since uptake of C₆-ceramide required $\approx$ 4 h (see below; Fig. 3), these results suggest a relatively rapid response of Rb to ceramide. The specificity of ceramide's

FIG. 2. Ceramide-induced dephosphorylation of Rb. (A) Dose-response experiment. MOLT-4 cells were preincubated in 10 ml (2×10^5 cells per ml) of RPMI 1640 medium supplemented with 2% FBS for 1–2 h before treatments with C₆-ceramide (C₆-Cer) for 4 h. C₆-ceramide was delivered in ethanol as indicated. (B) Time course. Similarly, treatments were done with 10 μM C₆-ceramide for the indicated times. (C) Structures of D-erythro-C₆-ceramide, D-erythro-dihydro-C₆-ceramide, and D-erythro-sphingosine.

effect was determined by using similar concentrations of the structurally related molecule dihydroceramide. This naturally occurring lipid differs from ceramide (and sphingosine) in lacking the double bond between carbons 4 and 5 of the sphingoid backbone although, similar to ceramide, it is acylated at the 2-amino position (Fig. 2C). After 4 h of treatment, dihydro-C₆-ceramide had minimal effects on Rb protein dephosphorylation at 10, 15, and 20 μM (data not shown), consistent with its lack of effects on cell growth (19, 29). Additionally, previous studies have shown (20) that other lipids including dioctanoylglycerol, stearylamine, dihydrosphingosine, palmitate, and oleate had no effect on Rb phosphorylation.

Since both sphingosine and ceramide are capable of inducing Rb dephosphorylation, it became necessary to determine whether ceramide's effects were independent of sphingosine or whether ceramide was simply acting as a precursor for intracellular sphingosine generation. C₆-[3-³H]Ceramide was used to study uptake and metabolism of exogenously added ceramide. We found that C₆-ceramide was rapidly taken up by cells (Fig. 3). C₆-Ceramide uptake was maximal ≈4 h after

FIG. 3. C₆-ceramide (C₆-Cer) uptake by MOLT-4 cells. Cells were treated with ³H-labeled C₆-ceramide, and uptake and metabolism were determined.

treatment and was sustained for >20 h. During this time, very little metabolism of C₆-ceramide was observed. Interestingly, [3-³H]sphingosine was not detectable. Thus, the effects of C₆-ceramide on the dephosphorylation state of Rb are independent of conversion to sphingosine.

The converging roles of ceramide and Rb on growth suppression led us to examine whether ceramide's ability to suppress growth is mediated through Rb. We compared the effect of ceramide on the proliferation of cells containing or lacking functional Rb. MOLT-4 cells contain functional Rb as shown by the formation of E2F–Rb complexes on gel shift analysis (data not shown). Therefore, MOLT-4 cells were treated with increasing concentrations of C₆-ceramide for 48 h. As is shown in Fig. 4A, cell growth was inhibited starting at 5 μM C₆-ceramide. Growth inhibition was dose-dependent and was maximal at 20 μM. In contrast, C₆-ceramide was remarkably less potent in inhibiting the proliferation of WERI-Rb-1 retinoblastoma cells, which lack Rb (30) (Fig. 4A).

In another approach, we examined the growth inhibiting property of ceramide in two cell lines with similar growth characteristics *in vitro* obtained from transforming mink lung epithelial cells with either wild-type TAg from simian virus 40 or a mutant TAg. Wild-type TAg is capable of binding Rb [predominantly the dephosphorylated form and other proteins such as the closely related protein p107 (31)] and thus sequestering it. Therefore, the cell line PVU-0, transformed using wild-type TAg, presumably lacks functional dephosphorylated Rb (32). The cell line, Mk-13, expresses the K1 mutant TAg that has lost the ability to bind Rb and, therefore, retains functional Rb (32). Treatment of these two cell lines with increasing concentrations of C₆-ceramide for 48 h resulted in very divergent effects. The Rb-containing cell line Mk-13 was very sensitive to C₆-ceramide with dose-dependent growth inhibition starting at 5 μM and becoming maximal at 25 μM (Fig. 4B). In contrast, the PVU-0 cell line was significantly

FIG. 4. Growth inhibitory effects of ceramide as a function of Rb status. (A) MOLT-4 cells (containing Rb) and WERI-Rb-1 cells (lacking Rb) were seeded in 10 ml of RPMI 1640 medium with 2% FBS at 2×10^5 cells per ml and treated with the indicated concentrations of C₆-ceramide delivered in ethanol vehicle. Trypan blue-excluding cells were counted by using a hemocytometer at 48–72 h. Data are the average of three experiments. (B) PVU-0 cells expressing wild-type large T antigen (TAg) and Mk-13 cells expressing the K1 mutant of TAg, which lacks Rb-binding capacity, were seeded at 5×10^4 cells per ml in 24-well plates in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The medium was changed to DMEM with 2% FBS 16 h later, and the cells were treated with C₆-ceramide as indicated. After 48 h, the cells were trypsinized, and viable cells were counted by using a hemocytometer. Data are the average of three experiments.

more resistant to growth inhibition by C₆-ceramide even at 50 μ M. Therefore, at least part of the growth inhibitory effects of ceramide appeared to require the presence of functional Rb. Cells that had functional Rb were very sensitive to the growth suppressive effects of ceramide, whereas cells in which Rb was inactivated genetically (WER1-Rb-1) or by complexing with TAg (PVU-0) were significantly more resistant.

We have shown that ceramide treatment of MOLT-4 cells results in a specific cell cycle arrest in the G₀/G₁ phase of the cell cycle (42). If Rb plays a role in mediating the effects of ceramide on cell cycle arrest, then interfering with Rb function should abrogate the effects of ceramide on cell cycle. We infected MOLT-4 cells with a retrovirus encoding the adenoviral E1A antigen, which binds Rb and inactivates it (33–35). Expression of E1A in the resulting cell line (MOLT-4-E1A) was determined by immunoblot analysis using a specific E1A antibody (Fig. 5A). Mock-infected MOLT-4-LXSN cells, not expressing E1A, were treated with increasing concentrations of C₆-ceramide for 18 h (Fig. 5B). There was a dose-dependent increase in the number of cells in the G₀/G₁ phase of the cell cycle with an increase from 46.9% in control cells to 68.4% in cells treated with 15 μ M C₆-ceramide. In contrast, MOLT-4-E1A cells when treated with similar concentrations of C₆-ceramide showed no increase in the number of cells in G₀/G₁ (Fig. 5C). In fact, there was a slight paradoxical decrease in the number of cells in G₀/G₁ and concomitant increase in cells in G₂/M. Therefore, the ability of ceramide to induce a specific cell cycle arrest in G₀/G₁ is only seen in cells containing functional Rb.

DISCUSSION

These studies demonstrate that ceramide is capable of inducing Rb dephosphorylation. The studies also suggest that a functional Rb is required to mediate ceramide-induced growth suppression based on (i) the relative resistance of Rb-lacking cells to growth suppression by ceramide, (ii) the ability of TAg (but not TAg mutated in the Rb-binding pocket) to prevent ceramide-induced growth suppression, and (iii) the ability of E1A to prevent ceramide-induced cell cycle arrest. Whereas none of these criteria are sufficient, combined they do suggest a role for Rb as a downstream effector.

These studies carry the following implications. (i) They provide a mechanistic basis for ceramide-induced growth arrest through dephosphorylation of Rb, which in turn is thought to play a critical role in regulation of cell cycle progression through the G₁/S check point (36). (ii) These studies provide strong evidence for a role for endogenous ceramide in mediating cell cycle arrest. This is supported by the significant and specific accumulation of endogenous ceramide in response to growth factor withdrawal, which, coupled to the acute and potent effects of ceramide on cell cycle progression, demonstrate the ability of endogenous ceramide to induce cell cycle arrest. (iii) The requirement for a functional Rb for mediating the effects of ceramide on cell cycle arrest supports the role of Rb as a downstream target for ceramide. Thus, these studies provide the foundations for a cell signaling pathway initiated at the cell membrane (activation of membrane sphingomyelinase) with generation of a lipid second messenger (ceramide) resulting in regulation of a major nuclear pathway of cell cycle suppression (i.e., Rb).

These studies also underscore the “specificity” of the action of ceramide. A major problem with the study of lipid second messengers has been the assumption that many of the effects, especially those on growth suppression, may be due to non-specific lipid effects. Whereas the specificity of ceramide has been established structurally by using a number of inactive analogs (19), the significant resistance of cells lacking Rb (or cells expressing viral proteins) to growth suppressor effects of ceramide underscores the mechanistically driven effects of ceramide on growth suppression.

Establishing a connection between ceramide and Rb raises a number of important future directions for investigation aimed at defining mechanisms of cell cycle regulation and their perturbation during oncogenesis. Thus, it is important to explore the universality of regulation of Rb by ceramide; that is, whether this is a specific event related to serum factor withdrawal or extends to other inducers of cell cycle arrest that operate through Rb dephosphorylation. It will also be important to determine the molecular mechanisms by which ceramide induces Rb dephosphorylation. In preliminary studies, dephosphorylation of Rb by ceramide was not affected by okadaic acid, a potent inhibitor of ceramide-activated protein phosphatase (12, 13, 37), or by activators of the protein kinase

FIG. 5. Cell cycle inhibition by ceramide is abrogated in cells expressing adenoviral E1A protein. (A) Western blot analysis of adenoviral Rb-binding protein, E1A, in MOLT-4-E1A (lane 2) and in control MOLT-4-LXSN cells (lane 1). (B) Ceramide-induced G₀/G₁ cell cycle arrest in MOLT-4-LXSN cells. Cells grown in 2% FBS were treated with ethanol vehicle or with C₆-ceramide as indicated for 18 h. (C) Lack of ceramide-induced cell cycle arrest in MOLT-4-E1A cells.

C pathway that are known to abolish the apoptotic events induced by ceramide (1).

In conclusion, ceramide may constitute an important component of the signaling mechanisms operating in regulation of Rb. More intriguingly, these studies suggest the hypothesis that perturbation in this ceramide-Rb tumor suppressor pathway may contribute to oncogenesis. It is already established that defects in Rb are present in some tumors (estimated at 5%) (38–41). As a corollary, defects in ceramide generation (i.e., proximal signaling events leading to activation of sphingomyelinase) or defects downstream of ceramide (e.g., ceramide-activated protein phosphatase) emerge as important candidates for oncogenic mechanisms.

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