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Stereoselectivity of Induction of the Retinoblastoma Gene Product (pRb) Dephosphorylation by D-*erythro*-Sphingosine Supports a Role for pRb in Growth Suppression by Sphingosine[†]

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ABSTRACT: Sphingosine has been shown to inhibit cell growth in many cell lines although the mechanism of this effect remains obscure. More recently, D-*erythro*-sphingosine has been shown to act as an early inducer of dephosphorylation of the retinoblastoma gene product (pRb) in the lymphoblastic leukemia cell line MOLT-4 [Chao, R., Khan, W., & Hannun, Y. A. (1992) *J. Biol. Chem.*, *267*, 23459−23462]. In the current study, the role of the natural D-*erythro*-sphingosine in regulation of cell growth and pRb dephosphorylation was evaluated using chemically synthesized pure isomers of sphingosine. Of the four possible stereoisomers of sphingosine, D-*erythro*-sphingosine was most active in inducing dephosphorylation of pRb protein with an $EC_{50\%}$ of 0.6 μM whereas its enantiomer L-*erythro*-sphingosine was 8-fold less potent with an $EC_{50\%}$ of 5 μM. The dose responses for inhibition of cell growth were nearly identical to the $EC_{50\%}$ for pRb dephosphorylation with D-*erythro*-sphingosine causing 50% inhibition at 0.6 μM whereas L-*erythro*-sphingosine was 5−6-fold less potent. All of the stereoisomers were taken up by the cells, and the greater potency of D-*erythro*-sphingosine was not due to differences in cellular uptake. The metabolism of D-*erythro*-sphingosine was also studied to evaluate the possible role of sphingosine metabolites on regulation of retinoblastoma protein. Evidence is provided against a role for ceramide or sphingosine 1-phosphate as mediators of the effects of sphingosine on pRb dephosphorylation. These results support a specific role for D-*erythro*-sphingosine in regulation of phosphorylation of pRb and provide evidence for a role of pRb dephosphorylation in mediating the growth inhibitory effects of sphingosine.

Sphingolipids constitute a diverse and ubiquitous group of membrane lipids (Hakomori, 1981). Extensive investigation over the last few decades has elucidated the structural complexity of sphingolipids and has identified multiple biologic and immunologic activities of these compounds (Hakomori, 1981; Hannun & Bell, 1989). With the recent discovery of inhibition of protein kinase C by sphingosine (Hannun et al., 1986; Merrill et al., 1986), attention was directed to a possible role for sphingolipids in signal transduction whereby sphingolipids serve as precursors to second messengers and intracellularly active molecules (Hannun & Bell, 1989; Merrill, 1991). Studies aimed at evaluating this hypothesis have resulted in identification of a sphingomyelin cycle in HL-60 leukemia cells (Okazaki et al., 1989; Hannun & Bell, 1993); these studies suggested a role for ceramide in regulation of cell proliferation and differentiation possibly through activation of a protein phosphatase (Hannun et al., 1993; Merrill, 1992).

Although multiple biological and biochemical activities of sphingosine have been described over the last few years, a physiologic function for free sphingosine has remained rather elusive (Merrill & Stevens, 1989; Hannun et al., 1991). Many of the demonstrated cellular activities of sphingosine occur in response to concentrations higher than endogenous levels of sphingosine, and where examined, the biochemical targets for sphingosine do not show much specificity for the natural stereoisomer (Merrill et al., 1989).

Recently, we have shown that sphingosine causes potent and early dephosphorylation of the retinoblastoma gene product (pRb) (Chao et al., 1992). pRb is a tumor suppressor phosphoprotein (Knudson, 1971; Friend et al., 1987; Fung et al., 1987; Lee et al., 1987), and multiple studies suggest that hypophosphorylated pRb is the active form that may function by causing a cell cycle arrest (Weinberg, 1991; Chellappan et al., 1991; Chen et al., 1989; DeCaprio et al., 1988; Mihara et al., 1989). The addition of sphingosine to MOLT-4 leukemia cells at concentrations of 0.2−1.0 μM resulted in dephosphorylation of pRb within 2−4 h (Chao et al., 1992). Therefore, it became important to determine whether the effects of sphingosine were specific for the natural stereoisomer and to investigate if the action of sphingosine was due to metabolism to other sphingolipids. In this study, the four stereoisomers of sphingosine were synthesized and characterized. We show that pRb dephosphorylation is most potently induced by the natural D-*erythro* stereoisomer. Moreover, the potency and selectivity of

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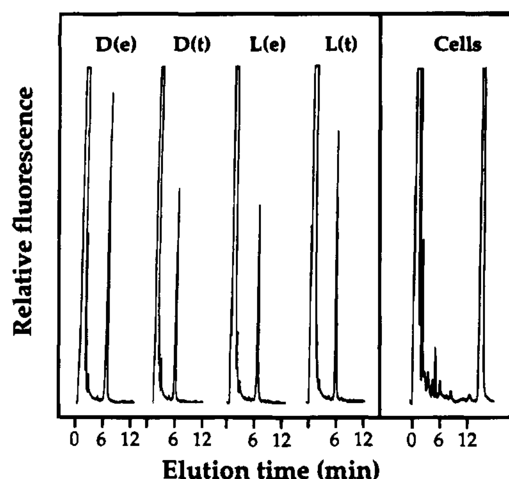

FIGURE 1: HPLC analysis of the four stereoisomers of sphingosine. Sphingosines were reacted with OPA and analyzed on HPLC as described (Merrill et al., 1988).

D-*erythro*-sphingosine on pRb dephosphorylation closely match the selectivity and potency of growth inhibition in response to sphingosine. The effects of sphingosine are shown to be unlikely due to further metabolism to either ceramide or sphingosine 1-phosphate, the major metabolites of sphingosine. The implications of these results on the physiologic role of sphingosine in regulating pRb dephosphorylation and the role of Rb in mediating the effects of sphingosine on cell growth are discussed.

MATERIALS AND METHODS

Preparation of the Four Stereoisomers of Sphingosine. D- and L-*erythro*- and D- and L-*threo*-sphingosines were prepared by stereoselective synthesis from either L- or D-serine (Nimkar et al., 1988). Products were purified by preparative TLC ($CHCl_3$/MeOH/$2NH_4OH$, 4:1:0.1 v/v). Purity of obtained sphingosines was established at >95% by TLC [detection by I_2 vapor, $KMnO_4$, and ninhydrin spray gives a single spot with characteristic differences in R_f value between *erythro* (R_f = 0.32) and *threo* (R_f = 0.29) forms], and according to synthesis there is no contamination of one enantiomer by another. Purity was also established at >98% by HPLC with precolumn OPA derivatization (Merrill et al., 1988) (HPLC solvent system: methanol/phosphate buffer, 9:1 v/v, flow rate 2.0 mL/min) (Figure 1). All structures were also verified by proton NMR.

Cell Culture. MOLT-4 cells (ATCC) were grown in RPMI-1640 with L-glutamine (Gibco) supplemented with 10% fetal bovine serum (Gibco) and pH adjusted with $NaHCO_3$ and incubated at 37 °C in 5% CO_2 and 90% humidity.

For proliferation studies, cells were resuspended at density 2×10^5 cells/mL in 10 mL of RPMI-1640 supplemented with 2% FBS. Cells were incubated for 1 h and subsequently treated with the indicated concentrations of sphingosine isomers dissolved in 10 μL of ethanol vehicle (ethanol had no effects on cell growth). At the indicated time points, viable cells (based on ability to exclude Trypan blue (Sigma)) were counted using a hemocytometer (Reichert).

Immunoblotting. Immunoblotting was performed as described (Chao et al., 1992). Briefly, following treatment of cells with sphingosine, cells were centrifuged, washed, and resuspended in 100 μL of 1 × PBS. A 5 μL aliquot of suspension was used for protein estimation, and the remaining 100 μL of suspension was added to 100 μL of 2 × sample buffer, which contained 4% instead 2% SDS, and was boiled for 7 min. Protein (40 or 50 μg/well) was applied to a 6% SDS−PAGE, then transferred to nitrocellulose paper, blocked with nonfat dry milk, and immunoblotted with anti-Rb antibodies (Pharmingen), followed by biotinylated anti-mouse IgG_1 antibodies (Amersham), and then streptavidin-conjugated horseradish peroxidase (Amersham). The filter was then color developed with 4 chloronaphthol (Bio-Rad).

pRb Phosphorylation Studies. The degree of pRb phosphorylation/dephosphorylation was determined by Western blotting as described (Chao et al., 1992) since SDS−PAGE resolves the slower migrating hyperphosphorylated forms of pRb from faster migrating hypophosphorylated forms (Buchkovich et al., 1989).

Sphingosine Uptake. MOLT-4 cells (2×10^5 cells/mL) were treated with 0.1 μM or 1 μM of each of the four stereoisomers of sphingosine in a total volume of 10 mL. At the indicated time points, cells were pelleted and lipids were extracted by the method of Bligh and Dyer (1959). Sphingosines were quantitated by HPLC analysis as described (Merrill et al., 1988).

D-erythro-Sphingosine Metabolism. [3-^{3}H]Sphingosine was used to study metabolism of D-*erythro*-sphingosine. MOLT-4 cell were preincubated in media supplemented with 2% fetal bovine serum and treated with 1 μM sphingosine (specific activity 0.3 mCi/μmol). Cells were collected at indicated times and washed, and lipids were extracted according to Folch and coauthors (Folch et al., 1957). Lipids were dried, resuspended in chloroform/methanol (10:1 v/v), and subjected to separation by thin layer chromatography in 3 solvent systems. System A: 1-butanol/acetic acid/water (3:1:1 v/v) (to separate sphingosine metabolites); system B: chloroform/methanol/28% ammonium hydroxide (4:1:0.1 v/v) (to separate ceramides); system C: chloroform/methanol/glacial acetic acid/water (50:30:8:4 v/v) (to separate phospholipids). Different lipids were used as standards to identify the products of sphingosine metabolism. After chromatography, plates were dried, developed in I_2, sprayed with Enhance spray (DuPont), and subjected to radiography for periods of time from 1 to 6 days. Spots containing radioactive compounds were scraped, and radioactivity was measured using a scintillation counter.

RESULTS

Effects of Stereoisomers of Sphingosine on pRb Dephosphorylation. To evaluate the effects of the four stereoisomers of sphingosine on Rb dephosphorylation, proliferating MOLT-4 cells were treated with each of the stereoisomers for 4 h. The status of pRb phosphorylation was examined by immunoblotting, since the hyperphosphorylated forms of pRb migrate in the SDS−PAGE slower than the hypophosphorylated forms. Treatment of MOLT-4 cells with increasing concentrations of D-*erythro*-sphingosine shifted the pRb protein from the predominantly hyperphosphorylated form observed in proliferating cells to progressively hypophosphorylated forms (Figure 2A). Initial effects of D-*erythro*-sphingosine were obtained at about 500 nM, with complete dephosphorylation occurring at concentrations of 0.75−1 μM (Figure 2A). The $EC_{50\%}$ (concentration causing 50% effect) was estimated at 0.6 μM (Table 1) using densitometric

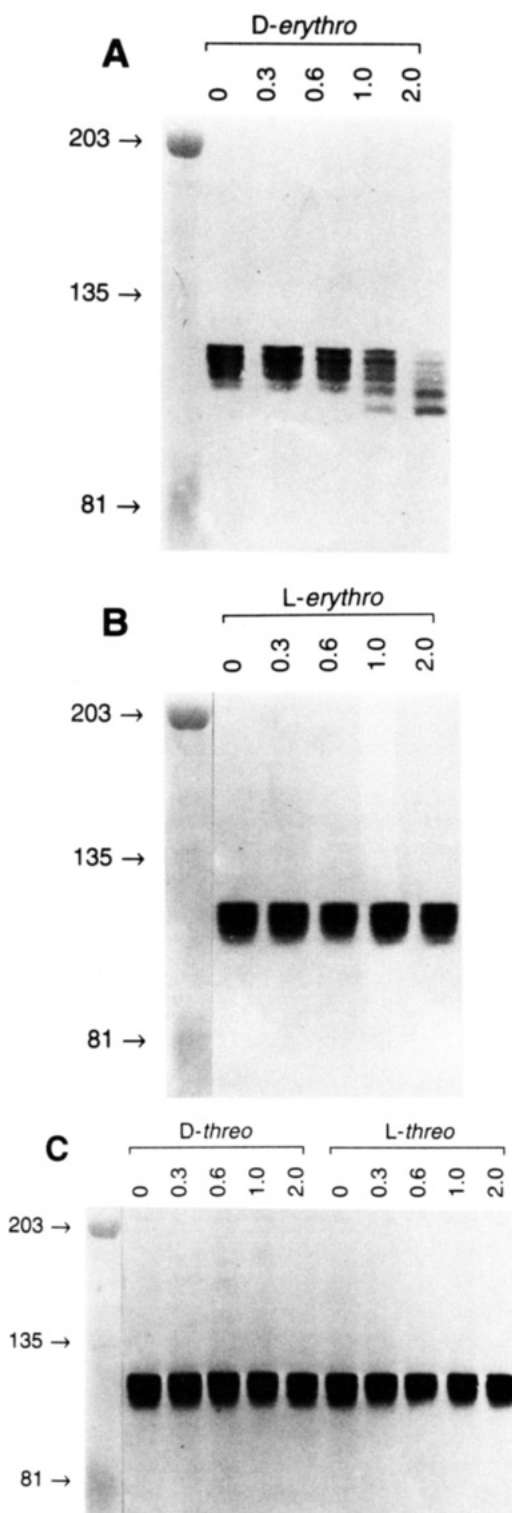

FIGURE 2: Induction of Rb dephosphorylation by sphingosine stereoisomers. MOLT-4 cells (2×10^5 cells/mL) were treated with the indicated concentrations of the four stereoisomers of sphingosine (final ethanol concentration 0.1% v/v) for 4 h. pRb phosphorylation was determined by Western blotting as indicated in the Materials and Methods where the least phosphorylated forms of pRb migrate fastest. Concentrations of sphingosines are indicated in micromolar quantities above the gels. The positions of Kaleidoscope (Bio-Rad) molecular weight standards are shown on the left. (A) D-*erythro*-Sphingosine; (B) L-*erythro*-sphingosine; (C) D-*threo*-sphingosine and L-*threo*-sphingosine.

analysis of developed blots. Significantly, L-*erythro*-sphingosine, the enantiomer of D-*erythro*-sphingosine, was approximately 8−10-fold less potent than the natural stereoisomer. Minimal dephosphorylation was observed at 2.5 μM, and partial dephosphorylation of pRb was obtained at 5 μM (Figure 2B, data not shown) with an $EC_{50\%}$ estimated at 5 μM (Table 1). Similarly, D-*threo*-sphingosine showed no activity at concentrations up to 2 μM (Figure 2C). At higher concentrations, this compound was significantly toxic (data not shown). L-*threo*-Sphingosine was also largely inactive up to 2 μM (Figure 2D). Thus, dephosphorylation of retinoblastoma protein appears to be selectively induced by D-*erythro*-sphingosine with significantly less potency of the enantiomeric L-*erythro*-sphingosine.

Table 1: Specificity and Potency of Effects of Sphingosine Isomers on pRb Dephosphorylation and Growth Inhibition

stereoisomer of sphingosine	pRb dephosphorylation, $EC_{50\%}$ (μM)[a]	cell growth inhibition, $IC_{50\%}$ (μM)	
		day 1	day 2
D-*erythro*	0.6	0.6	0.6
L-*erythro*	5.0	3.7	3.2
D-*threo*	>2.5	3.2	2.0
L-*threo*	>1.0	1.8	1.0

[a] Data were calculated from densitometric analysis of Western blots.

Effects of Sphingosine Stereoisomers on Cell Proliferation. Since pRb has been postulated to play a role in regulating cell cycle progression (Weinberg, 1991; Chen et al., 1989; Mihara et al., 1989), the ability of sphingosine to induce pRb dephosphorylation and to cause a G_0/G_1 arrest in cell cycle progression suggested that pRb may indeed play a role in mediating the effects of sphingosine on cell growth inhibition. Therefore, we reasoned that the specificity and potency of action of sphingosine on pRb dephosphorylation should be closely matched by similar specificity and potency on growth inhibition if pRb dephosphorylation is mechanistically related to growth inhibition. Proliferating MOLT-4 cells were treated with increasing concentrations of the four stereoisomers of sphingosine, and cell growth was monitored over 3 days. D-*erythro*-Sphingosine inhibited cell growth at even the lowest concentration tested (0.5 μM). Higher concentrations of D-*erythro*-sphingosine caused complete inhibition of cell growth followed by cell death within 1−2 days (Figure 3A). From dose−response studies (Figure 4A), the $IC_{50\%}$ for growth inhibition was estimated at 0.6 μM, very closely matching the $EC_{50\%}$ for induction of retinoblastoma protein dephosphorylation (Table 1). L-*erythro*-Sphingosine, the enantiomer of the natural D-*erythro*-sphingosine, was much less active in inhibiting growth, with initial effects observed at 2.5 μM and complete inhibition of cell growth and cell death occurring at 5 μM (Figure 3B) with an $IC_{50\%}$ estimated at 3.7 μM (Figure 4A and Table 1). Similarly, the unnatural stereoisomer D-*threo*-sphingosine was less potent than D-*erythro*-sphingosine (Figure 3C) with significant cytotoxicity observed at 5 μM. The dose response of growth inhibition (Figure 4B) resulted in an estimated $IC_{50\%}$ of 2.2 μM (Table 1). As with pRb dephosphorylation, L-*threo*-sphingosine showed intermediate activity, with growth inhibition at concentrations ranging between 1 and 2.0 μM (Figure 3D) with the dose response (Figure 4B) demonstrating an $IC_{50\%}$ of 1.8 μM at day 1 and 1 μM at day 2 (Table 1). It should be noted that sphingosine isomers displayed a relatively narrow range of effective concentrations, suggesting cooperativity of action. The data as summarized in Table 1 show a very close correlation between the $EC_{50\%}$ for

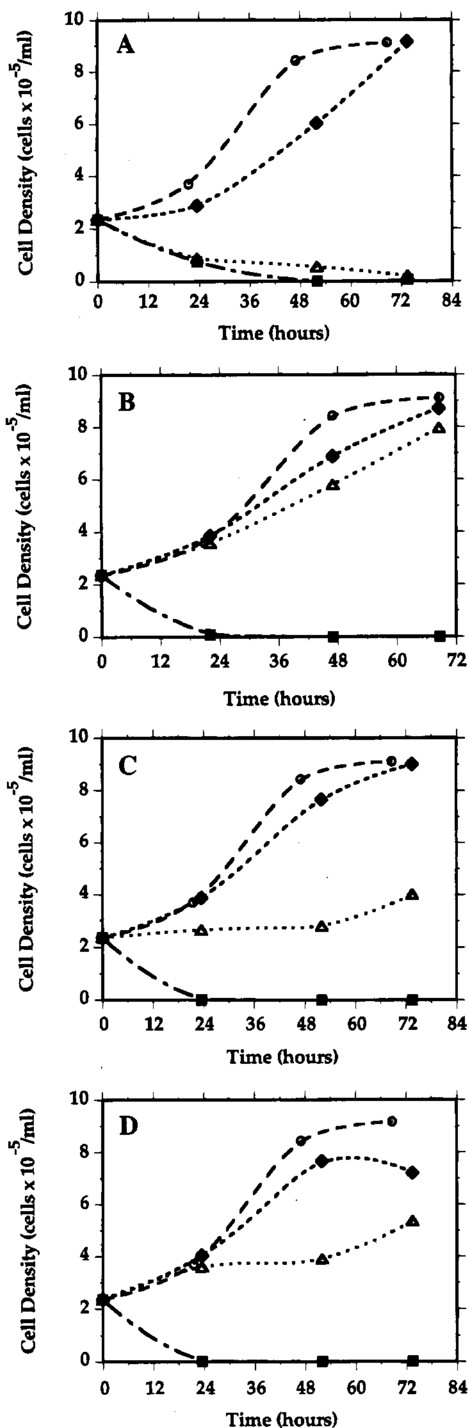

FIGURE 3: Time dependence of inhibition of cell growth by sphingosine stereoisomers. MOLT-4 cells (2 × 10^5 cells/mL) were treated with the indicated concentrations of sphingosine, and cells were counted at days 1, 2, and 3. Control cells received an equal concentration of ethanol (0.1% v/v). (A) D-*erythro*-Sphingosine: (○) ethanol, (♦) 0.5 μM, (△) 0.75 μM, and (■) 1 μM sphingosine; (B) L-*erythro*-sphingosine: (○) ethanol, (♦) 1.0 μM, (△) 2.5 μM, and (■) 5 μM sphingosine; (C) D-*threo*-sphingosine: (○) ethanol, (♦) 1.0 μM, (△) 2.5 μM, and (■) 5 μM sphingosine; (D) L-*threo*-sphingosine: (○) ethanol, (♦) 0.75 μM, (△) 1.0 μM, and (■) 2.5 μM sphingosine.

induction of pRb dephosphorylation and the $IC_{50\%}$ for growth inhibition by the stereoisomers of sphingosine.

Cellular Levels of Sphingosine in Control and Treated Cells. The uptake and levels of the four stereoisomers of sphingosine were determined by HPLC analysis following OPA derivatization (Figure 5). MOLT-4 cells were preincubated in RPMI media supplemented with 2% fetal bovine serum for 1−2 h before treatment. Stereoisomers of sphingosine (0.1 μM) were added as ethanol solution. All of the stereoisomers were taken up rapidly from the culture medium (i.e., had achieved a near-maximum level of 35−80 pmol/10^6 cells within minutes). The maximum uptake of D-*erythro*-sphingosine was achieved at 30 min after treatment, and at 1 h D-*erythro*-sphingosine was already significantly metabolized (Figure 5). Over 24 h there was significant metabolism of D-*erythro*- and L-*threo*-sphingosine, intermediate metabolism of D-*threo*-sphingosine, and very little metabolism of L-*erythro*-sphingosine (Figure 5). The uptake of sphingosine was dependent on the concentration of sphingosine used for treatment: when 1 μM sphingosine was used there was an increase in sphingosine uptake (achieving maximum of 1.2 nmol/10^6 cells within 30 min

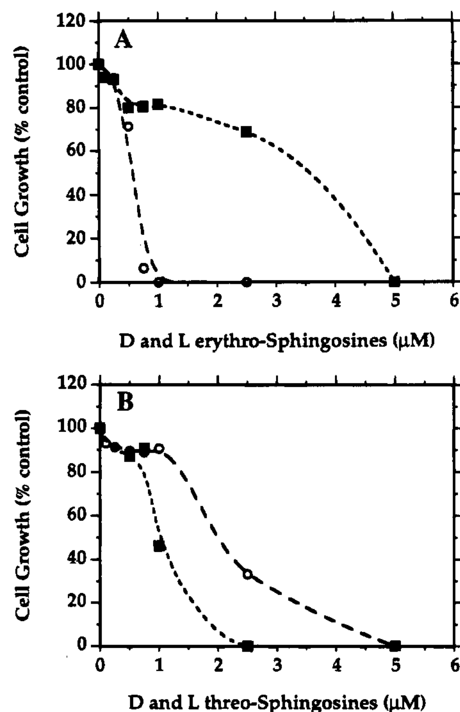

FIGURE 4: Dose−response of growth inhibition by sphingosine stereoisomers. MOLT-4 cells (2 × 10^5 cells/mL) were treated with the indicated concentrations of sphingosine stereoisomers, and cells were counted at 2 days following treatment. (A) Dependence on concentration of (○) D-*erythro*- and (■) L-*erythro*-sphingosine; (B) dependence on concentration of (○) D-*threo*- and (■) L-*threo*-sphingosine.

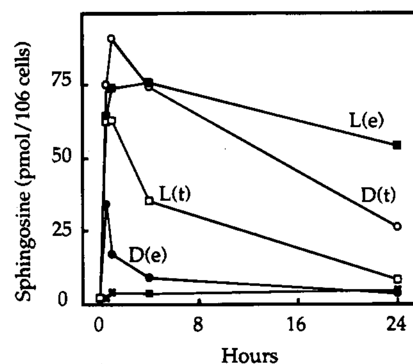

FIGURE 5: Cellular uptake of long-chain basis. MOLT-4 cells were incubated with 0.1 μM of each of the four stereoisomers of sphingosine, and cellular levels were determined using OPA derivatization as described in Materials and Methods.

for D-*erythro*-sphingosine) but the kinetics of intracellular sphingosine changes were very much the same as presented in Figure 5 (data not shown). Thus D-*erythro*-sphingosine achieved the lowest cellular levels for the duration of the the experiment. These results, therefore, argue against a role for enhanced uptake as a mechanism for its increased potency compared to the other stereoisomers.

Metabolism of Exogenous Sphingosine. As is evident in Figure 5, exogenous sphingosine underwent extensive metabolism. Therefore, it became important to evaluate the possible role of major metabolites of sphingosine (ceramide and sphingosine 1-phosphate) as potential mediators of pRb dephosphorylation. To investigate metabolic pathways of sphingosine, [3-^{3}H]-D-*erythro*-sphingosine was used. The experiments with labeled sphingosine showed similar results as direct measurement of sphingosine content: sphingosine was taken up by MOLT-4 cells rapidly. The analysis of metabolic products showed that sphingosine underwent two major metabolic pathways: sphingosine was degraded through the sphingosine 1-phosphate pathway, and it was also metabolized to ceramide and more complex sphingolipids (sphingomyelin, cerebrosides, and others) (Figure 6). A significant fraction (25−30%) of sphingosine was acylated to ceramide (Figure 7), causing a doubling in ceramide levels. This increase is unlikely to result in effects on pRb dephosphorylation, since treatment of cells with sphingomyelinase increased ceramide levels (3−5-fold), but did not affect pRb phosphorylation status (data not shown).

The Potentiation of Sphingosine Effects by Fumonisin B_1. Another successful approach to separate sphingosine and ceramide effects on pRb dephosphorylation which we used was utilization of fumonisin B_1, an inhibitor of reaction of sphingosine acylation to ceramide. Fumonisins have been shown as specific inhibitors of sphingolipid metabolism by blocking *de novo* synthesis of sphingolipids at the step of acylation of sphinganine to dihydroceramide (Wang et al., 1991). Fumonisins also are potent inhibitors of sphingosine acylation to ceramide (Merrill et al., 1993). Initially, fumonisin B_1 was tested for its effects on cell growth. MOLT-4 cells treated with increasing concentrations of fumonisin B_1 (up to 100 μM) for 24 h exhibited a growth pattern similar to that of the control (ethanol treated) cells (Figure 8, and data not shown). Thus, at least over 24 h of treatment, fumonisin B_1 did not affect growth of MOLT-4 cells (similar results were obtained for HL-60 cells; data not shown). However, cotreatment of cells with fumonisin B_1 and sphingosine demonstrated that the growth inhibitory effects of sphingosine were greatly potentiated by fumonisin B_1. Sphingosine (1 μM) alone inhibited cell growth by 40% as compared to control cells over a period of 24 h of treatment. Cotreatment of MOLT-4 cells with 1 μM sphingosine and 20 μM fumonisin B_1 resulted in 65% inhibition of cell growth as was measured by trypan blue exclusion. Fumonisin B_1 (100 μM) together with 1 μM sphingosine resulted in 80% inhibition of cell growth (Figure 8). Thus, fumonisin B_1 potentiated growth inhibitory effects of sphingosine by blocking conversion of sphingosine to ceramide. Pretreatment of MOLT-4 cells even with 10 μM fumonisin B_1 slowed down metabolism of exogenous sphingosine as well as inhibited formation of ceramide (data not shown).

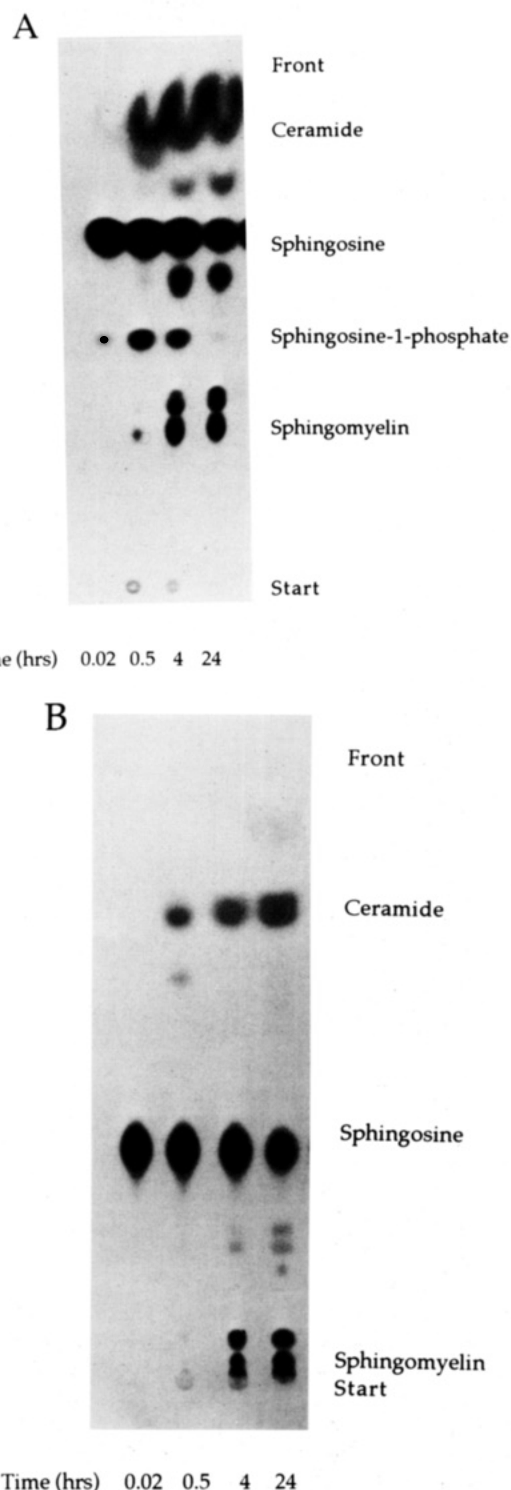

FIGURE 6: Metabolism of sphingosine. MOLT-4 cells were incubated with 1 μM [3-^{3}H]sphingosine, and extracted lipids were subjected to TLC separation in (A) 1-butanol/acetic acid/water (3:1:1 v/v) and (B) chloroform/methanol/28% ammonium hydroxide (4:1:0.1 v/v) systems as described in Materials and Methods.

Because fumonisin B_1 potentiated sphingosine effects on cell growth inhibition, we tested fumonisin B_1 as a modulator of pRb phosphorylation. Fumonisin B_1 alone did not affect pRb phosphorylation. Cotreatment of cells with 1 μM sphingosine and 10 μM fumonisin B_1 did not change phosphorylation status of pRb. However, 50 μM of fumonisin B_1 potentiated dephosphorylation of retinoblastoma protein which was induced by 1 μM sphingosine (Figure 9).

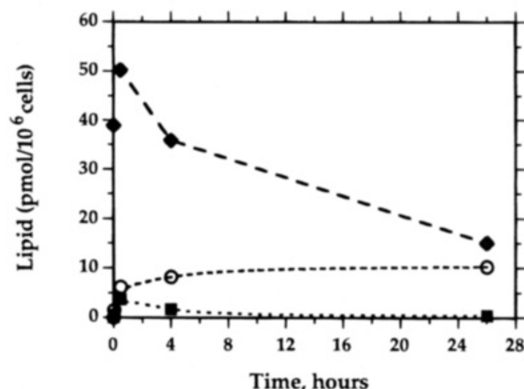

FIGURE 7: Sphingosine metabolism. MOLT-4 cells were incubated with 1 μM [3-^{3}H]sphingosine as described in Materials and Methods for indicated times, and extracted lipids were subjected to TLC separation. Lipid spots were scraped and radioactivity was measured. (♦) Sphingosine, (○) ceramide, (■) sphingosine 1-phosphate. Data are representative of two independent experiments.

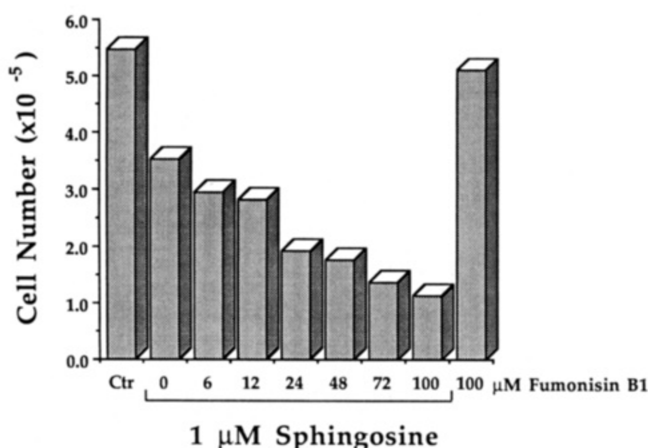

FIGURE 8: Effects of sphingosine and fumonisin B_1 on MOLT-4 cell growth. MOLT-4 cells were treated with the indicated concentrations of fumonisin B_1 in the presence or absence of 1 μM sphingosine for 24 h. Experiments were carried out in RPMI media supplemented with 2% fetal bovine serum. Data are representative of two independent experiments.

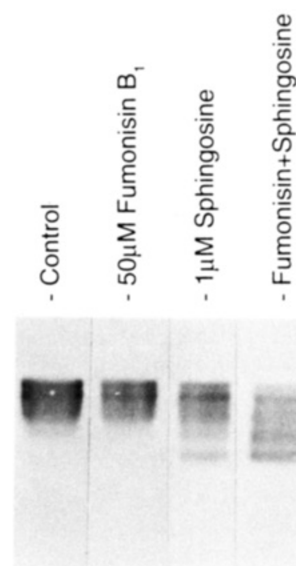

FIGURE 9: Effects of sphingosine and fumonisin B_1 on Rb protein phosphorylation. MOLT-4 cells were pretreated with 50 μM fumonisin B_1 for 4 h and then were treated with 1 μM sphingosine or a corresponding amount of ethanol for an additional 4 h. Western blot was carried out as described in Materials and Methods.

Thus, fumonisin B1 potentiates sphingosine effects probably due to inhibition of sphingosine acylation. These results argue against a role for ceramide (and subsequent metabolites) in mediating the effects of sphingosine.

Sphingosine 1-Phosphate Effects on pRb Phosphorylation. Study of sphingosine metabolism showed that sphingosine 1-phosphate is formed rapidly. Therefore, sphingosine 1-phosphate became another possible candidate which induces retinoblastoma protein dephosphorylation. We tested sphingosine phosphate on its ability to modulate phosphorylation of pRb. Concentrations up to 10 μM of sphingosine phosphate neither affected pRb phosphorylation nor modulated sphingosine effects (data not shown).

DISCUSSION

The results from this study carry two major implications relating to the role of pRb in mediating the effects of sphingosine on cell growth and to the possible role of sphingosine as a regulator of pRb function.

Multiple lines of evidence implicate pRb as a mediator of the effects of exogenous sphingosine in induction of growth inhibition. First, the potency of sphingosine in inducing pRb dephosphorylation closely matches the potency of sphingosine in inhibiting cell growth such that the 50% effective concentration in both situations is approximately 0.6 μM (Table 1). Second, the structural selectivity of sphingosine in inducing pRb dephosphorylation also closely matches that for growth inhibition such that D-*erythro*-sphingosine demonstrates the greatest activity, with its enantiomer L-*erythro*-sphingosine showing the least activity. L-*threo*-Sphingosine demonstrates intermediate activity. Third, in other studies, we find that sphingosine does not cause specific growth inhibition in the WERI cell line which lacks a functional retinoblastoma protein.[1] In this cell line, concentrations of sphingosine up to 5 μM were without effect. Fourth, sphingosine induces a G_0/G_1 arrest in cell cycle progression, and pRb has been implicated in regulating the transition from G_0/G_1 to S phase. Moreover, the phosphorylation status of pRb has been correlated with this activity such that the dephosphorylated form of pRb predominates in G_0/G_1 and the hyperphosphorylated form predominates in S and G_2/M (Weinberg, 1991; Chellappan et al., 1991; Chen et al., 1989; Goodrich et al., 1991). This has led to the hypothesis that phosphorylation of pRb in late G_0/G_1 results in release of a block and allows cells to progress through the cell cycle (Weinberg, 1991; Goodrich et al., 1991). Therefore, induction of pRb dephosphorylation by sphingosine offers a plausible mechanism for the ability of sphingosine to inhibit cell growth and to induce an arrest in cell cycle progression.

Equally as important, these data provide further evidence for the importance of pRb phosphorylation/dephosphorylation in regulation of cell growth since previous studies have relied primarily on correlating the status of pRb phosphorylation with cell growth. On the other hand, the use of sphingosine allows for the unique opportunity to induce early dephosphorylation of pRb and examine the biological consequences since sphingosine is one of the few agents known to induce early dephosphorylation of pRb that precedes growth arrest.

Moreover, the significant toxicity observed with D-*erythro*-sphingosine and the other sphingosine isomers in this study

[1] G. Dbaibo and Y. A. Hannun, manuscript in preparation.

raises the possibility that cell death may be a consequence of total dephosphorylation of pRb. According to this hypothesis, moderate pRb dephosphorylation (e.g., as observed with 0.5 μM D-*erythro*-sphingosine) would result in growth inhibition while higher degrees of dephosphorylation (as observed with higher concentrations D-*erythro*-sphingosine) may result in cell death.

In CHO cells, evidence was provided that dihydrosphingosine (sphinganine) and sphingosine induce cytotoxicity by a protein kinase C-dependent mechanism (Stevens et al., 1990). On the other hand, in 3T3 fibroblasts, sphingosine has been shown to exert a mitogenic effect (primarily determined by thymidine uptake) in a protein kinase C-independent mechanism (Zhang et al., 1990). Thus, sphingosine may have a dual effect on cell growth depending on the cell type, and these effects may be mediated by multiple pathways.

The results from this study also raise the possibility that D-*erythro*-sphingosine may function as an endogenous regulator of pRb function. The effects of sphingosine on pRb dephosphorylation show a selectivity for the natural D-*erythro* stereoisomer as opposed to its enantiomer the L-*erythro* form. This stereoselectivity suggests that D-*erythro*-sphingosine may interact with an endogenous cellular protein since proteins are able to discriminate between various stereoisomers. Such a protein either could be a direct target of sphingosine involved in signal transduction/cell regulation or could be an enzyme that specifically metabolizes D-*erythro*-sphingosine into other bioactive derivatives. The latter possibility seems to be somewhat unlikely at this point since none of the major metabolites of sphingosine (sphingosine 1-phosphate or natural C_{18}-ceramide) or putative sphingosine metabolites (such as sphingosylphosphorylcholine or sphingomyelin) are able to induce pRb dephosphorylation. The early effects of sphingosine on pRb dephosphorylation are unlikely to be due to acylation of sphingosine to ceramide, since an increase in ceramide level after incubation of MOLT-4 cells with bacterial sphingomyelinase did not affect pRb phosphorylation (similar results were observed for HL-60 cells; data not shown). Thus, an increase in intercellular levels of ceramide cannot be responsible for early dephosphorylation of pRb.

One wonders how this system can show selectivity for D-*erythro*-sphingosine without exhibiting a strict specificity for one stereoisomer. This may be due to two factors. First, all four stereoisomers present the same orientation of the groups on carbons 1−3 if the orientation of the alkyl chain is varied (Merrill & Stevens, 1989). Therefore, the stereoisomer selectivity may reflect the ability of a target protein to discriminate between long-chain (sphingoid) bases that differ in their structures at positions 3−5. Evidence for this has already surfaced for pRb in the greater potency of sphingosine (with a 4,5 *trans* double bond) versus sphinganine (with saturated carbons at positions 4 and 5) (Chao et al., 1992). Second, because only one stereoisomer of the long-chain bases is encountered in nature, there has not been a selective pressure in evolution to force proteins to discriminate between the four stereoisomers.

The relationship between these findings with *exogenously* added sphingosine to the possible regulation of pRb phosphorylation by *endogenous* sphingosine is obscure. On the surface, the amount of sphingosine that has been taken up from the medium is much higher than the endogenous levels. This comparison is not valid, however, if endogenous sphingosine is located in a specific subcellular compartment where it could affect pRb phosphorylation more effectively than exogenous long-chain bases. At present, the best evidence that sphingosine may have an effect on pRb phosphorylation that is biologically relevant is the stereoselectivity for the D-*erythro* isomer.

At this point, two possibilities emerge as to the potential physiologic regulation of retinoblastoma protein by sphingosine. According to the first possibility, sphingosine may act as a classical second messenger whereby short-term elevations in sphingosine levels may result in pRb dephosphorylation. Another attractive possibility is that long-term changes in sphingosine levels (i.e., over several hours) may allow sphingosine to act as a sensor for extracellular growth conditions such that significant elevations in sphingosine may result in pRb dephosphorylation. Further studies are required to monitor sphingosine levels under different growth conditions and correlate these levels with phosphorylation status of pRb.

In conclusion, the results from this study provide evidence for a cellular pathway of cell regulation whereby D-*erythro*-sphingosine induces pRb dephosphorylation resulting in cell growth inhibition. The selectivity and potency of D-*erythro*-sphingosine also support the hypothesis that D-*erythro*-sphingosine may function as an endogenous regulator of pRb dephosphorylation and cell growth.

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