Cell-cycle-dependent changes in ceramide levels preceding retinoblastoma protein dephosphorylation in G_2/M

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Ceramide functions as a growth-inhibitory lipid-signalling molecule and might have a role in mediating the effects of extracellular agents on cell growth, differentiation and senescence. Here we investigate the roles of ceramide in cell cycle progression. With the use of the model of serum withdrawal, we were able to synchronize Wi-38 human diploid fibroblasts at different stages of cell cycle. Serum stimulation resulted in G_0 to G_1/S progression as determined by flow cytometric analysis and [^{3}H]thymidine incorporation. Analyses of endogenous ceramide levels demonstrated that ceramide levels remained relatively constant on serum stimulation, indicating that ceramide might not be critical during G_1/S transition. Treating exponentially growing Wi-38 human diploid fibroblasts with nocodazole led to cell cycle arrest at the G_2/M phase of the cell cycle; 2 h after the removal of nocodazole, retinoblastoma (Rb) protein became dephosphorylated and the cells exited from G_2/M and moved to the G_1 phase of the new cycle. When cells were released from G_2/M block by nocodazole, and before Rb protein dephosphorylation, endogenous ceramide levels transiently increased up to 2-fold at 0.5 h after the removal of nocodazole. Fumonisin B1, an inhibitor of ceramide synthase, inhibited the elevation of ceramide levels. Desipramine and SR33557, both acid sphingomyelinase inhibitors, did not have an appreciable effect on the elevation of ceramide levels. Furthermore, fumonisin B1 inhibited Rb protein dephosphorylation induced by endogenous ceramide but not by exogenous ceramide. These results demonstrate for the first time changes in ceramide during cell cycle progression and suggest that ceramide synthesized *de novo* might function as an endogenous modulator of Rb protein and cell cycle progression.

INTRODUCTION

Numerous studies have demonstrated that sphingolipids have an important role in signal transduction and cell growth regulation. Once activated by extracellular agents such as 1,25-dihydroxycholecalciferol (vitamin D_3), tumour necrosis factor α and interferon γ, sphingomyelinase hydrolyses sphingomyelin and forms ceramide and phosphocholine [1–3]. Ceramide then acts as a lipid second messenger to mediate the effects of extracellular agents on cell growth, differentiation, apoptosis and cellular senescence.

Ceramide has previously been shown to activate a protein phosphatase (ceramide-activated protein phosphatase) *in vitro* [4], induce retinoblastoma (Rb) protein dephosphorylation [5], induce programmed cell death [6] and act as a mediator of cellular senescence [7].

Ceramide has also been shown to induce a G_0/G_1 arrest in cell cycle progression in multiple cell lines. Exposure of Molt-4 cells to C_6-ceramide produced a dose-dependent and early cell cycle arrest [8]. Exposure of NIH 3T3 cells overexpressing insulin-like growth factor 1 receptors to *threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol (PDMP), a glucosylceramide synthase inhibitor that leads to increased cellular ceramide concentration, resulted in an arrest of the cell cycle at both G_1 and G_2/M transitions [9]. Exposure of Wi-38 human diploid fibroblasts (HDF) to C_6-ceramide arrested cells at both the G_1 and G_2/M phases of cell cycle (Lee, J. Y., Bielawska, A. E., Leohardt, L. G. and Obeid, L. M., unpublished work). These findings lend support to a postulated role of ceramide in cell cycle progression.

We have shown previously [7] that ceramide levels increase severalfold in senescent HDF when compared with exponentially growing cells. Because senescent cells seem to be arrested in both the G_0/G_1 and G_2/M phases of cell cycle, we investigated the role of ceramide in cell cycle progression. In the present study we found that ceramide levels did not change when Wi-38 HDF were synchronized at the G_0/G_1, G_1/S or G_2/M phases. We demonstrate for the first time that endogenous ceramide levels transiently increased when cells were released from a G_2/M block, and before Rb protein dephosphorylation. In addition we provide evidence that synthesis of sphingolipids *de novo* might contribute to the transient increase of ceramide after the release of cells from a nocodazole block. Further, inhibition of Rb protein dephosphorylation by fumonisin B1 has led us to propose that ceramide might be an important endogenous modulator of Rb protein and cell cycle progression.

EXPERIMENTAL

Materials

Wi-38 HDF were obtained from the NIA Aging Cell Repository (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco Laboratories (Gaithersburg, MD, U.S.A.). Fetal bovine serum (FBS) was supplied by Summit Biotechnology (Fort Collins, CO, U.S.A.). [^{3}H]Thymidine was from ICN. DNase-free RNase, nocodazole, propidium iodide and Desipramine were from Sigma (St. Louis, MO, U.S.A.). Fumonisin B1 was a gift from Dr. Marc Mumby (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). SR33557 was a gift from Dr. Jean-Pierre Jaffrezou (National Center for Scientific Research, Claudius Regaud

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HDF, human diploid fibroblasts; PDMP, *threo*-1-phenyl-2-decanoylamino-3-morpholinopropan-1-ol; Rb, retinoblastoma.

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Center, Toulouse, France). D-*erythro*-C_6-ceramide was a gift from Dr. Alicja Bielawska (Duke University, Durham, NC, U.S.A.). Rb protein antibody was purchased from Pharmingen (San Diego, CA, U.S.A.). The biotinylated rabbit anti-(mouse IgG_1) antibodies were from Amersham Life Science (Arlington Heights, IL, U.S.A.) and the streptavidin-conjugated horseradish peroxidase and related reagents were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Cell culture

Cells were maintained in DMEM supplemented with 4.5 g/l glucose and 10 % (v/v) FBS under air/CO_2 (19:1) at 37 °C in a humidified incubator. For experiments, cells were normally seeded at 6×10^5 cells per plate and were fed after 2 days.

Cell cycle synchronization

Exponentially growing Wi-38 cells were arrested in G_0/G_1 by incubation in 0.1 % (v/v) FBS-supplemented DMEM for 48–72 h. To obtain an S-phase cell population, G_0/G_1 arrested cells were re-fed with 10 % (v/v) FBS-supplemented DMEM for various durations. Populations of cells arrested in mitotic phase were prepared by incubation of cells in 0.4 μg/ml nocodazole for 16–18 h. To release the cells from the mitotic block, cells were washed with FBS and incubated in complete DMEM for up to 12 h.

Thymidine incorporation

Cells were incubated with 0.5 μCi/ml [^{3}H]thymidine for the last 4 h of incubation before harvesting. The cells were then harvested and precipitated by the addition of 5 % (w/v) trichloroacetic acid. The incorporation of [^{3}H]thymidine into cell precipitates was quantified by scintillation counting.

Flow cytometry analysis

After different treatments, cells were harvested by treatment with trypsin and resuspended in PBS, fixed in ethanol and stored at −20 °C as described elsewhere [8,10]. On the day of analysis, cells were washed with PBS and treated with 20 μg/ml DNase-free RNase A at 37 °C for 30 min. Cells were then stained with 100 μg/ml propidium iodide for 5–10 min and analysed with a FACStar^{plus} flow cytometer (Becton-Dickinson, San José, CA, U.S.A.).

Ceramide measurements

Cells were harvested and lipids were extracted as described [11], then assayed for ceramide by the diacylglycerol kinase method [12], normalized to total cellular phospholipid [13] and represented as pmol of ceramide/nmol of lipid phosphorus.

Western blotting

After the indicated durations of different treatments, Wi-38 cells were harvested on ice. Proteins were fractionated by SDS/PAGE [6 % (w/v) gel] as described by Laemmli [14]. After SDS/PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane [15]. The nitrocellulose membrane was blocked in 5 % (w/v) dried milk in PBST [0.1 % (v/v) Tween 20 in 1 × PBS], incubated with Rb protein antibody, and then either developed with an enhanced chemiluminescence detection system (Amersham Corp.) or detected with biotinylated rabbit anti-(mouse IgG_1) and streptavidin-conjugated horseradish peroxidase [7,16].

RESULTS

Levels of endogenous ceramide during G_1/S transition

Ceramide has been shown to induce Rb protein dephosphorylation and inhibit cell growth. It was our interest to examine the levels of endogenous ceramide in different phases of the cell cycle. It has been established that perturbations of normal cells such as serum withdrawal induces cell cycle arrest and/or cell death. To examine the role of ceramide in cell cycle progression, we first synchronized Wi-38 HDF at the G_0/G_1 phase of cell cycle by serum deprivation. The Wi-38 cell line was chosen for this study because it is a non-transformed cell line and undergoes cellular senescence in culture. Wi-38 HDF were serum-deprived for 72 h; they were then serum-stimulated. Cells were harvested at various time points after serum stimulation for analysis of the DNA distribution (Table 1) by flow cytometry and [^{3}H]thymidine uptake (Figure 1). Table 1 shows that after 72 h of serum withdrawal (defined as zero time) 90 % of the cells were arrested in G_0/G_1 as determined by flow cytometry, whereas less than 10 % of the cells were in S phase and in G_2/M phase as expected. Cells began to enter S phase approx. 18 h after serum stimulation. The maximal effect was seen at 24 h after serum stimulation. At

Table 1 Synchronization of Wi-38 HDF by serum deprivation

Wi-38 HDF were first arrested at the G_0/G_1 phase by serum withdrawal for 72 h (defined as zero time); this was followed by serum stimulation. Cells were harvested at various time points after serum stimulation for analysis of the DNA distribution. Results are expressed as means ± S.D.

	Cells in phase (% of total)		
Time (h)	G_0/G_1	S	G_2/M
0	88.9 ± 3.70	9.6 ± 4.15	1.6 ± 0.40
0.5	96.4 ± 1.70	3.0 ± 2.25	0.7 ± 0.60
3	96.8 ± 1.65	2.4 ± 1.70	0.9 ± 0.10
6	96.4 ± 0.95	2.5 ± 1.15	1.2 ± 0.20
9	93.4 ± 0.95	5.4 ± 0.80	1.3 ± 0.05
12	93.0 ± 1.50	5.8 ± 1.40	1.2 ± 0.05
15	92.4 ± 2.50	6.9 ± 2.65	0.8 ± 0.15
18	90.5 ± 0.00	8.2 ± 0.05	1.5 ± 0.05
21	83.4 ± 1.05	15.7 ± 1.15	1.0 ± 0.10
24	68.8 ± 0.65	21.4 ± 1.95	9.0 ± 1.25
27	68.2 ± 1.60	12.7 ± 0.5	19.1 ± 1.05
30	67.8 ± 0.95	13.5 ± 2.55	18.8 ± 1.60

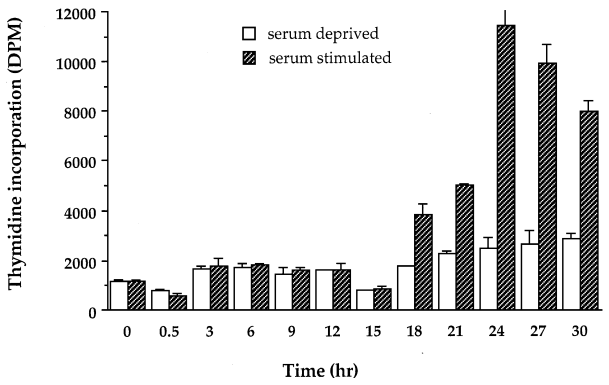

Figure 1 [^{3}H]Thymidine uptake profile after serum deprivation and stimulation

Cells were able to undergo [^{3}H]thymidine incorporation into newly synthesized DNA in response to serum stimulation. Results represent one experiment performed in duplicate.

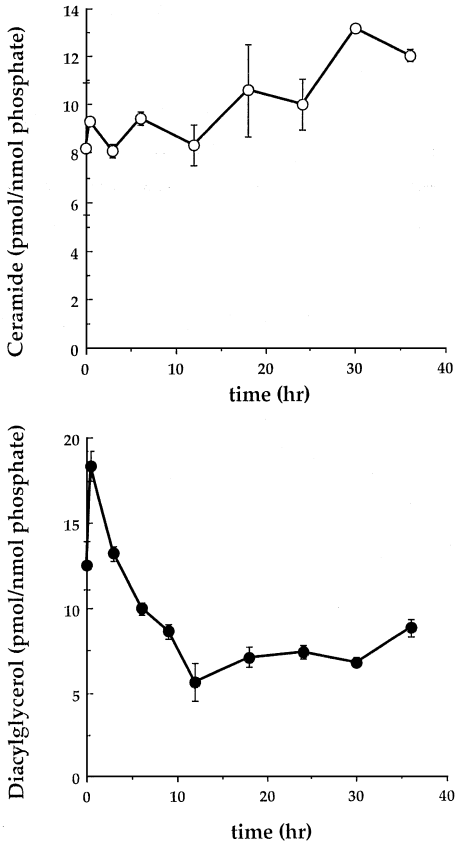

Figure 2 Endogenous ceramide levels do not change at different stages of the cell cycle

Wi-38 HDF were grown and treated as for Figure 1. Both serum-deprived and serum-stimulated cells were harvested at the indicated time points. Lipids were extracted and assayed for endogenous ceramide (upper panel) and diacylglycerol (lower panel) levels. The results are representative of two experiments performed in duplicate.

that time approx. 20% of the cells were in S phase, 20% of the cells were in G_2/M and 60% of the cells were in G_1 phase, which is similar in distribution to exponentially growing cells. The [^{3}H]thymidine uptake profile showed similar results, with cells beginning to synthesize DNA 18 h after serum stimulation and reaching a level similar to exponentially growing cells 24 h after serum stimulation (Figure 1). These results were in accordance with widely established sychronization conditions.

Ceramide levels were then determined by diacylglycerol kinase assay as described in the Experimental section. Figure 2 (upper panel) shows that endogenous ceramide levels did not change significantly in the transition from the arrested state to the cycling state. These results suggest that ceramide does not have a role in G_1/S transition. Interestingly, the levels of diacylglycerol increased significantly immediately (30 min) after serum stimulation, as we demonstrated previously [17], then returned to the basal levels 3 h later and stayed constant throughout the period examined (Figure 2, lower panel). Therefore these results illustrate that cells are responsive to serum stimulation and that diacylglycerol levels respond early to serum stimulation.

Synchronization of Wi-38 HDF at G_2/M phase

A second check point in the cell cycle is G_2/M phase, when cells exit from mitosis and start new rounds of cycling. Rb protein becomes dephosphorylated as cells exit from G_2/M to enter the new G_1 phase of the cell cycle [18,19]. Because ceramide induces Rb protein dephosphorylation, and the levels of ceramide increased somewhat at the time point when cells were moving in and out of G_2/M (between 24 and 30 h after serum stimulation) (Table 1; Figure 2, upper panel), we wondered whether ceramide has a role at the G_2/M transition. When cells were synchronized by serum deprivation, the synchrony was lost after serum stimulation for an extended period of time and it was difficult to capture Rb protein dephosphorylation as the cells exited mitosis. We elected to use DNA inhibitory agents to arrest the cells at different stages of the cell cycle, with an emphasis on cells in G_2/M. Nocodazole, a reversible inhibitor of microtubule polymerization, has been reported to arrest the cells in the mitotic metaphase [20,21]. We therefore employed nocodazole in our next series of experiments.

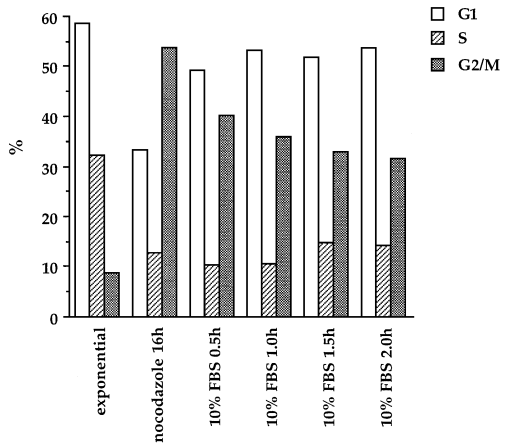

Figure 3 Effects of nocodazole block and release on cell cycle

Exponentially growing Wi-38 HDF were treated with 0.4 μg/ml nocodazole for 16–18 h. Cells were washed with PBS and then incubated with medium containing 10% (v/v) FBS for the indicated periods. Cells were arrested at the G_2/M phase after nocodazole treatment and were able to enter the G_1 phase after the removal of nocodazole. Results are representative of four experiments.

Exponentially growing cells were incubated with 0.4 μg/ml nocodazole for 16–18 h, and DNA distribution was analysed by flow cytometry. Approx. 50% of the cells were arrested in the G_2/M phase of the cell cycle after treatment with nocodazole (Figure 3). When cells were released from the G_2/M block by exposure to medium containing 10% (v/v) FBS, most of the arrested cells gradually returned to the cycling state. However, treatment with nocodazole caused a small population of cells to be locked in the G_2/M phase (Figure 3). Nevertheless nocodazole provided an excellent tool for studying the biology of cell cycle progression.

Dephosphorylation of Rb protein as cells exited from G_2/M phase

To determine whether Rb protein became dephosphorylated as the cells exited from mitosis in our model system, the phosphorylation status of Rb protein was examined. Both hyperphosphorylated and hypophosphorylated forms of Rb protein were detected in exponentially growing cells as well as in the cells arrested at G_2/M as expected (Figure 4). After release of the cells from the G_2/M block, the hypophosphorylated form of Rb protein appeared as early as 1 h (results not shown) and became predominant at 2 h, consistent with the cells moving into the G_1 phase of the new cycle (Figure 4).

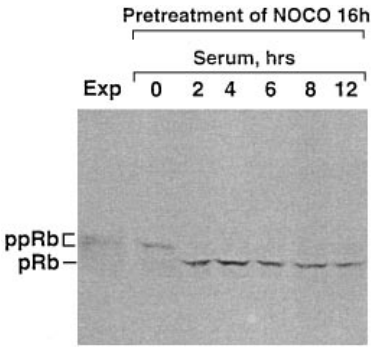

Figure 4 Effects of nocodazole block and release on Rb protein phosphorylation/dephosphorylation status

Exponentially growing Wi-38 HDF were treated as for Figure 3. Cells were harvested at the indicated time points. Proteins were subjected to Western blot analysis with Rb protein antibody and detected by biotinylated rabbit anti-(mouse IgG_1) and streptavidin-conjugated horseradish peroxidase as described in the Experimental section. Rb protein became dephosphorylated as the cells exited from the G_2/M phase of the cell cycle.

Levels of endogenous ceramide increased as cells exited from G_2/M before Rb protein dephosphorylation

We next examined whether endogenous ceramide levels change at this checkpoint and before Rb protein dephosphorylation. We therefore determined endogenous ceramide levels under the above conditions. Interestingly, ceramide levels underwent a 2-fold increase between 0.5 and 1 h after the cells were released from G_2/M arrest (Table 2). To demonstrate that the change in ceramide levels was not restricted to Wi-38 cells, we also examined the change in ceramide levels in NIH 3T3 cells. We found a similar change in ceramide levels on release from G_2/M block in NIH 3T3 cells comparable with that in Wi-38 cells (Table 2). Thus the change in ceramide levels observed in Wi-38 cells was not restricted to this cell type.

Mechanisms of changes in ceramide level during cell cycle and the effect of ceramide on Rb protein

We next examined whether synthesis of sphingolipids *de novo* contributes to the transient elevation of ceramide after the release of the cells from a nocodazole block. Fumonisin B1, an inhibitor of sphingosine N-acyltransferase or ceramide synthase [22], inhibited the elevation of ceramide levels (Table 3), suggesting that synthesis of sphingolipids *de novo* might contribute to the transient increase of ceramide after the release of cells from a nocodazole block. Desipramine, which induces the proteolytic degradation of acid sphingomyelinase [23], did not show a significant effect on the elevation of ceramide levels (Table 2). SR33557, a potent acid sphingomyelinase inhibitor [24], also did not have an appreciable effect on the elevation of ceramide levels (results not shown). These results suggest that neutral sphingomyelinase might be responsible for the transient increase in ceramide levels. Furthermore fumonisin B1 inhibited Rb protein dephosphorylation through endogenous ceramide (Figure 5, upper panel) but not through exogenous ceramide (Figure 5, lower panel), suggesting that ceramide might function as an endogenous modulator of Rb protein.

Table 3 Effects of fumonisin B1 on ceramide levels as cells exited from G_2/M block

Wi-38 HDF were grown and treated with nocodazole for 16–18 h as for Figure 3. Cells were washed with PBS and then incubated with medium containing 10% (v/v) FBS in the absence or presence of 25 μM fumonisin B1 for the indicated periods. For some cells, fumonisin B1 (25 μM) was added for 1 h before the removal of nocodazole. Ceramide levels were determined by the diacylglycerol kinase method and are expressed as the percentage change over nocodazole treatment. The results are representative of two different experiments.

Condition	Ceramide concentration (% of control)
Nocodazole, 16 h	100
0% FBS, 0.5 h	110
10% FBS, 0.5 h	170
10% FBS/fumonisin B1, 0.5 h	60
10% FBS/Desipramine, 0.5 h	170
0% FBS, 1.0 h	160
10% FBS,1.0 h	140
10% FBS/fumonisin B1, 1.0 h	60
10% FBS/Desipramine, 1.0 h	170

DISCUSSION

The results from this study demonstrate for the first time an elevation in endogenous ceramide levels during cell cycle progression. This increase occurs as cells exit from G_2/M to the G_1 phase of the cell cycle and is significantly more than increases seen in most other systems. Moreover this increase in ceramide levels precedes the dephosphorylation of Rb protein that occurs as the cells exit from a G_2/M block. In contrast, no changes in ceramide were observed during transitions from G_0 to G_1 to S to G_2/M. These results support and extend our previous biochemical studies demonstrating that ceramide induces Rb protein dephosphorylation in multiple cell lines [5,7]. These findings also suggest a role for endogenous ceramide in the transition from G_2/M to G_1.

Table 2 Endogenous ceramide levels increased before Rb dephosphorylation when cells were released from G_2/M block

Wi-38 HDF and NIH3T3 cells were grown and treated with nocodazole as for Figure 3. Cells were harvested, then lipids were extracted and assayed for levels of endogenous ceramide. Ceramide levels increased 2-fold between 0.5 and 1 h after release from G_2/M arrest by nocodazole, compared with exponentially growing and nocodazole-treated cells in Wi-38 HDF. The results are representative of three separate experiments.

Wi-38 cells		NIH 3T3 cells	
Condition	Ceramide concentration (pmol/nmol of P_i)	Condition	Ceramide concentration (pmol/nmol of P_i)
Exponential	2.10 ± 0.30	Exponential	17.8 ± 4.50
Nocodazole, 16 h	0.91 ± 0.19	Nocodazole, 13 h	8.30 ± 4.40
10% FBS, 0.5 h	3.96 ± 0.72	10% FBS, 15 min	19.9 ± 0.35
10% FBS, 1.0 h	2.49 ± 0.47	10% FBS, 30 min	29.4 ± 1.06
10% FBS, 2.0 h	1.59 ± 0.18	10% FBS, 45 min	17.6 ± 6.00

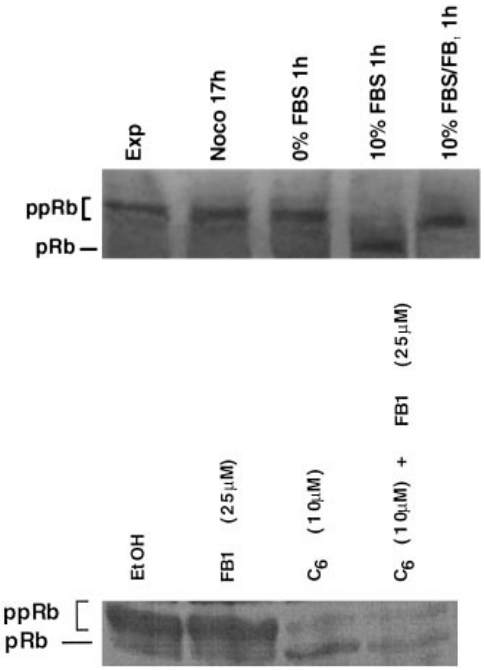

Figure 5 Effects of fumonisin B1 on Rb protein phosphorylation/dephosphorylation

Upper panel: Wi-38 HDF were grown and treated with nocodazole for 16–18 h as for Figure 3. Cells were washed with PBS and then incubated with medium containing 10% (v/v) FBS in the absence or presence of 25 μM fumonisin B1 for the indicated periods. For some cells, fumonisin B1 (25 μM) was added for 1 h before the removal of nocodazole. The degree of Rb protein phosphorylation was determined by Western blotting. Lower panel: Wi-38 HDF were grown and treated with 10 μM C_6-ceramide in the absence or presence of 25 μM fumonisin B1 for 16 h. The degree of Rb protein phosphorylation was determined as for the upper panel. Abbreviation: EtOH, ethanol. The results are representative of three independent experiments.

Several parameters begin to suggest a role for endogenous ceramide in the transition from G_2/M to G_1. First, we have shown previously that ceramide inhibits the phosphorylation of Rb protein in Wi-38 and Molt-4 cells [7,25]. Secondly, exogenous ceramides cause the dephosphorylation of Rb protein in cycling cells [5,7]. Thirdly, ceramide causes a cell cycle arrest in G_0/G_1 in Molt-4 cells and in Wi-38 HDF [8]. Fourthly, a functional Rb protein is required for ceramide-induced Rb protein dephosphorylation [5]. Fifthly, PDMP regulates endogenous ceramide and arrests cells at the G_0/G_1 and G_2/M phases of the cell cycle [9]. Lastly, our current studies on the endogenous levels of ceramide show significant but reversible changes that precede the transient dephosphorylation of Rb protein seen as cells move from G_2/M to G_1. The elevation of ceramide levels was also observed in NIH 3T3 cells (Table 2), indicating that the change in ceramide levels is not limited to one cell type. These results raise the possibility that the endogenous elevation of ceramide levels in the transition from G_2/M to G_1 might cause the activation of Rb protein by dephosphorylation. This might be essential for cells to progress to G_1.

It has been reported that changes in ceramide levels occur in response to an exchange of medium [26]. We have noticed that ceramide levels increased immediately (30 min) in cells in response to 10% (v/v) FBS compared with 0% FBS. However, ceramide levels also increased (60 min) after exposure to 0% FBS (Table 3). Additionally, cells were locked in the G_2/M phase as Rb protein remained in the hyperphosphorylated form in response to 0% FBS (Figure 5, upper panel). These results suggest to us that the change in ceramide levels is not simply due to a change of medium.

These results raise the question of the mechanism of the transient elevation in ceramide levels. By using fumonisin B1, an inhibitor of ceramide synthase, we found that the elevation of ceramide levels after the release of the cells from the G_2/M block was altered by the treatment with fumonisin B1. In addition, the fact that acid sphingomyelinase inhibitors Desipramine and SR33557 were ineffective in blocking the elevation of ceramide suggests that neutral sphingomyelinase might lead to the generation of ceramide after the release of the cells from the G_2/M block. Finally, fumonisin B1 is able to prevent Rb protein dephosphorylation induced by endogenous ceramide, but not by exogenous ceramide. These results established a cause–effect relationship between ceramide elevation and Rb protein dephosphorylation and added to our knowledge on the role of sphingolipids in cell cycle progression.

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