Activation of a retinoblastoma-protein-dependent pathway by sphingosine

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The retinoblastoma protein (Rb) is a tumour suppressor that is activated by dephosphorylation the function of which appears to be mediated, at least partly, through the inhibition of several transcription factors, such as E2F. We have recently described sphingosine, a sphingolipid-breakdown product, as a potent and specific inducer of Rb dephosphorylation resulting in inhibition of cell growth and a specific arrest in the G_0/G_1 phase of the cell cycle. Here we examine the role of Rb and its interaction with E2F in mediating the effects of sphingosine on cell growth. Sphingosine potently inhibited growth of lymphoblastic leukaemic cells, Molt-4, at submicromolar concentrations but showed a 10-fold reduced potency in inhibiting growth of retinoblastoma cells, WERI-Rb-1, which lack functional Rb. In addition, sphingosine's ability to inhibit growth of mink lung epithelial cells was significantly attenuated in cells overexpressing

simian virus 40 large T antigen which binds Rb and related proteins. Sphingosine treatment of Molt-4 cells, but not WERI-Rb-1 cells, resulted in the loss of the specific E2F bands produced by the interaction of E2F and its specific DNA sequence element on gel-shift assays. The concentration (submicromolar) and kinetics (4 h) of sphingosine treatment were identical with those required to induce Rb dephosphorylation. In addition, at similar concentrations, sphingosine caused *c-myc* down-regulation in Molt-4 cells starting at 6 h after treatment. These results demonstrate that activation of Rb by sphingosine leads to sequestration of E2F by the active (hypophosphorylated) form of Rb with the resultant loss of its DNA-binding and gene-transcribing abilities. A functional Rb is required to mediate the specific effects of sphingosine on growth arrest.

INTRODUCTION

Sphingolipid-breakdown products are emerging as important mediators of cell regulation. The recent discovery of the sphingomyelin cycle was an important initial step in elucidating the role of membrane sphingolipids in signal transduction. Evidence is accumulating to support a role for ceramide, the product of sphingomyelin hydrolysis, as a second messenger in this distinct pathway of signal transduction [1,2].

Multiple biological functions have been ascribed to another sphingolipid-breakdown product, sphingosine. These include inhibition of human platelet aggregation [3,4], inhibition of blood coagulation [5], inhibition of neutrophil activation [6], inhibition of cell differentiation [7], stimulation of mitogenesis [8] and others (reviewed in [9,10]). Many, but not all, of these effects appear to be due to the ability of sphingosine to potently and reversibly inhibit protein kinase C (PKC) [3].

Sphingosine modulates targets in addition to PKC, including activation of phosphatidic acid phosphohydrolase [11]. Recent studies have disclosed the ability of sphingosine to specifically and potently induce early dephosphorylation of the retinoblastoma protein (Rb) [12]. Rb is one of the most intensely studied examples of a growing list of tumour-suppressor proteins [13,14]. Loss of both copies of the *RB1* gene through deletion or mutation is associated with unrestrained cell growth and tumour formation. Tumours associated with loss of Rb function include childhood retinoblastoma and various osteosarcomas, bladder carcinomas, breast carcinomas and others [15,16]. Rb appears to play an important role in cell-growth suppression and regulation of cell-cycle progression [17–20]. The status of Rb phosphorylation is an important regulator of its activity. The hypophosphorylated form predominates in the G_0/G_1 phase of the

cell cycle [21]. Rb then becomes phosphorylated on multiple sites near the G_1/S transition, undergoes additional phosphorylation before mitosis, then is dephosphorylated at the end of mitosis [21,22]. Data accumulated so far indicate that the hypophosphorylated form of Rb is the active form [17]. It is this form that has been shown to bind (and possibly sequester) the transcription factor E2F [23], thus inhibiting its transcriptional activity [24]. The hypophosphorylated form of Rb is also a target for a direct physical interaction with the viral transforming proteins E1A of adenovirus, T antigen of simian virus 40 (SV40), and E7 of human papilloma virus [22,25–29]. The transforming ability of these viral oncoproteins correlates with their ability to interact with Rb. Phosphorylation of Rb results in its inactivation, reversal of its cell-cycle-blocking action and resumption of cell growth [19,30].

Little is known about the physiological regulators of Rb phosphorylation. Recent studies have shown that cyclins A, E, D2 and D3 when overexpressed are capable of inactivating Rb by inducing its phosphorylation [30,31]. However, the signalling pathways resulting in activation of Rb (by its dephosphorylation) have been largely undetermined.

In a previous study [12] we showed that sphingosine was effective in inducing Rb dephosphorylation at concentrations between 0.5 and 2.5 μ M, with the earliest effect detected at 2 h and peaking at 4 h. The effect was specific to sphingosine, in that dihydrosphingosine and other related long-chain amino bases were ineffective at inducing Rb dephosphorylation. Moreover, the effect of sphingosine was independent of PKC, which is inhibited by sphingosine at higher concentrations. Sphingosine concentrations effective at inducing Rb dephosphorylation were also capable of inducing growth inhibition of Molt-4 leukaemia cells and their specific arrest in the G_0/G_1 phase of the cell cycle.

Abbreviations used: Rb, retinoblastoma protein; PKC, protein kinase C; FBS, fetal bovine serum; SV40, simian virus 40; DMEM, Dulbecco's modified Eagle's medium; 1 $\times$ SSC, 0.15 M NaCl/0.015 M sodium citrate; 1 $\times$ Denhardt's, 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA.

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In this study we explore the functional effects of Rb dephosphorylation by sphingosine and the role of Rb in mediating the growth-inhibitory function of sphingosine. We show that sphingosine specifically inhibits the growth of cells containing functional Rb but not cells lacking functional Rb. The sphingosine-induced dephosphorylation of Rb results in the loss of E2F binding to its specific DNA sequence. In addition, sphingosine causes the down-regulation of *c-myc* mRNA which is dependent on E2F binding to its specific motif in the *myc* promoter. These findings begin to define an Rb-dependent pathway of cell regulation that can be activated by sphingosine.

MATERIALS AND METHODS

Materials

Stearylamine and dioctanoylglycerol were purchased from Sigma. Cyclin A antibody was a gift from Dr. James Schwarz (The Howard Hughes Medical Institute, Duke University). Rb antibody was purchased from Oncogene Science. *D-erythro*-Sphingosine and *L-erythro*-sphingosine were prepared by Alicja Bielawska as described [32].

Cell culture

Molt-4 (human lymphoblastic leukaemia; ATCC) and WERI-Rb-1 (retinoblastoma; ATCC) cells were grown in RPMI 1640 (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS). Mink lung epithelial cells transformed with either wild-type (PVU-0) or mutant (Mk-13) large T antigens of SV40 virus (a gift from Dr. Jonathan Horowitz, Duke University Medical Center) were grown in Dulbecco's modified Eagle's medium DMEM (Gibco) supplemented with 10% (v/v) FBS. Cell-growth experiments were performed in the same medium but supplemented with 2% (v/v) FBS. Sphingosine or stearylamine were delivered in ethanol at the indicated concentrations, and the final concentration of ethanol was always < 0.1%. Cell viability was evaluated by Trypan Blue exclusion.

Whole cell extracts

Cells (10^7) were washed once in ice-cold PBS then suspended in 100 μ l of lysis buffer [50 mM Hepes, pH 7.9, 250 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 0.1% Nonidet P40, 10% (v/v) glycerol, 4 mM NaF, 4 mM Na_3VO_4 , 1 mM dithiothreitol, 0.5 mM PMSF, 5 μ g/ml aprotinin and 1 μ g/ml pepstatin]. The cells were extracted on ice for 30 min then ultracentrifuged at 100000 g at 4 °C for 20 min. Aliquots of the supernatant were stored at -80 °C. Protein concentration was determined using the Bio-Rad assay.

E2F electrophoretic mobility-shift assay

Reactions were performed in a total volume of 20 μ l, using 2–5 μ g of protein from whole cell extracts in the presence of 1 μ g of sheared salmon sperm DNA, 70 μ g of BSA, 2.5% Ficoll and 0.1 ng of Klenow-labelled adenovirus E2 promoter probe [33] (a gift from Dr. James Schwarz). Final concentrations for the binding buffer were: 20 mM Hepes, pH 7.9, 40 mM KCl, 1 mM MgCl_2 , 0.1 mM EGTA, 0.4 mM dithiothreitol. Antibodies or unlabelled oligonucleotides were added as indicated. The reaction mixture was incubated for 20 min at room temperature. Equal amounts of the reactions were loaded on 4% non-denaturing polyacrylamide gels in 0.6 $\times$ TBEGTA (using EGTA instead of EDTA in standard Tris-borate-EDTA) and run at 300 V and 4 °C for 90 min.

Northern-blot analysis

Treated cells were harvested and washed twice with PBS. Total cellular RNA was isolated using a modification of the method of Chomczynski and Sacchi [34]. Total cellular RNA was fractionated on a 1% agarose denaturing gel containing formaldehyde. RNA was transferred to nitrocellulose blots, air-dried and then baked at 80 °C for 2 h. Blots were then hybridized using a hybridization solution containing 50% formamide, 2 $\times$ SSC, 50 mM Na_2PO_4 , 0.1% SDS, 10 $\times$ Denhardt's solution and sheared salmon sperm DNA at 400 μ g/ml where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate and 1 $\times$ Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA. Radiolabelled probes were synthesized using nick-translated fragments of vector Spt6T7-M4 containing exon III of the *c-myc* gene. Total RNA loaded on the gel was assessed using a nick-translated GC-rich cDNA probe which strongly hybridizes to 28S RNA. Both of these cDNA vectors in addition to Spt6T7-M3 (containing exon I of MYC) were gifts from Dr. Spence McCachren (Department of Medicine, Duke University). Hybridization proceeded for 16–24 h at 42 °C. Blots were then rinsed twice in 2 $\times$ SSC/0.2% SDS for 30 min at room temperature, twice in 0.2 $\times$ SSC/0.2% SDS at 65 °C and once in 0.1 $\times$ SSC/0.1% SDS. The nitrocellulose filters were autoradiographed using Kodak X-AR films at -70 °C with intensifying screens.

RESULTS

D-erythro-Sphingosine inhibits the growth of Rb-containing but not Rb-deficient cells

To investigate the role that Rb plays in mediating the growth-inhibitory effects of sphingosine, we compared the effects of sphingosine on proliferation of the Rb-containing Molt-4 cells and Rb-deficient WERI-Rb-1 cells. Sphingosine caused a dose-dependent inhibition of Molt-4 cell growth at concentrations between 0.5 and 1 μ M, the same concentration range that induces Rb dephosphorylation [12] (Figure 1). Stearylamine, a long-chain amino base structurally related to sphingosine (Figure 2), did not affect cell growth within the same concentration range

Figure 1 Effects of sphingosine and stearylamine on the growth of Molt-4 cells

Molt-4 cells were suspended at a density of 2×10^5 cells/ml in RPMI medium supplemented with 2% (v/v) FBS and treated with either sphingosine (○) or stearylamine (●) at the indicated concentrations. Control cells were treated with ethanol vehicle. Viability was determined by the ability to exclude Trypan Blue. Viable cells were counted after 48 h with a haemocytometer. Data are means $\pm$ S.E.M. of four different experiments.

Figure 2 Structures of *D*-erythro-sphingosine, *L*-erythro-sphingosine and stearylamine

Figure 3 Effect of sphingosine and stearylamine on growth of WERI-Rb-1 cells

WERI-Rb-1 cells were suspended in RPMI medium supplemented with 2% (v/v) FBS at a density of 2×10^5 cells/ml and treated with either sphingosine (○) or stearylamine (●) at the indicated concentrations. Control cells were treated with ethanol vehicle. Viable, Trypan-Blue-excluding, cells were counted using a haemocytometer after 48 h. Data are means $\pm$ S.E.M. of three different experiments.

(Figure 1). In contrast, in WERI-Rb-1 cells, sphingosine was unable to inhibit growth in the 0.5–2.5 μ M range (Figure 3). However, concentrations 5–10-fold higher achieved partial growth inhibition (Figure 3). Moreover, in this cell line, stearylamine was equally effective at producing growth inhibition under the same conditions (Figure 3). Therefore the specific growth-inhibitory effects observed at low concentrations of sphingosine were only seen in the cell line containing functional Rb. In the Rb-negative cell line, long-chain amino bases inhibited growth at significantly higher concentrations and without specificity for sphingosine.

As Molt-4 and WERI-Rb-1 cell lines differ in origin and may have important differences other than the presence or absence of functional Rb, definitive conclusions could not be made and a different approach was used. In this approach, we utilized the ability of the large T antigen of SV40 to bind to Rb (mainly in its dephosphorylated state) and its related proteins (e.g. p107). We studied the effect of sphingosine on the growth of two cell lines generated from transforming mink lung epithelial cells with either wild-type large T antigen or a mutant large T antigen

Figure 4 Sphingosine-induced growth inhibition in transformed mink epithelial cells with or without functional Rb

PVU-0 cells (●) transformed with large T antigen, thus lacking functional Rb, and Mk-13 cells (○) transformed with a mutant large T antigen incapable of binding Rb and its related proteins were seeded in DMEM and 10% FBS at 5×10^4 cells/ml in 24-well plates. The medium was changed to DMEM with 2% FBS 16 h later and the cells were treated with sphingosine at the indicated concentrations. Cells were treated with trypsin, and Trypan Blue-excluding cells were counted 48 h later using a haemocytometer. Data are means $\pm$ S.E.M. of three different experiments.

unable to bind Rb or its related proteins. The cell line PVU-0, transformed using wild-type large T antigen, contains Rb that has been sequestered, and thus inactivated, by large T antigen. The cell line Mk-13, transformed using the K1 mutant large T antigen, contains functional Rb, and related proteins, that were not complexed with the mutant T antigen. Treatment of Mk-13 cells, containing intact Rb, with increasing concentrations of sphingosine for 48 h resulted in a dose-dependent inhibition of cell growth (Figure 4). Treatment of PVU-0 cells with similar concentrations of sphingosine caused significantly less inhibition of growth observed at very high concentrations (Figure 4).

Therefore the growth-inhibitory effects of sphingosine were seen in cell lines containing functional Rb but not in cell lines where Rb was rendered inactive either through mutation or deletion (WERI-Rb-1) or by complexing with large T antigen (PVU-0).

***D*-erythro-Sphingosine inhibits E2F–DNA binding activity**

The cellular transcription factor E2F is an important target of Rb [22], and has been shown to interact directly with Rb. The status of Rb phosphorylation appears to influence the formation of the Rb–E2F complex which in turn regulates the transcriptional activity of E2F [24,35,36]. Therefore induction of Rb dephosphorylation by sphingosine may result in modulation of the function and activity of E2F. Initially we examined the effects of sphingosine on the DNA-binding activity of E2F complexes in gel mobility-shift assays. Using a probe derived from the adenovirus E2 promoter, three specific complexes were detected in growing Molt-4 cells, that represent distinct E2F–DNA binding activities (Figure 5a). These complexes were competed off by incubation with excess unlabelled oligonucleotide that contains a specific E2F binding sequence (Figure 5a, lane 2) but not with an excess of a similar oligonucleotide that contains a mutation in the E2F binding sequence (Figure 5, lane 3). The fastest migrating band, E2F–Rb, contains Rb in complex with E2F, as it was eliminated by addition of a monoclonal Rb antibody (Figure 5a, lane 4). Complexes E2F–cyclin A-1 and E2F–cyclin A-2 contain

Figure 5 Effect of *D-erythro*-sphingosine on E2F-DNA binding in Molt-4 cells

(a) Specificity of gel-shift complexes in Molt-4 cells. Whole-cell extracts and binding reactions were performed as described in the Materials and methods section in the presence of labelled probe alone (labelled WT; lane 1) or with excess unlabelled oligonucleotide containing the E2F-binding sequence (cold WT; lane 2), excess unlabelled oligonucleotide with a mutation in the E2F-binding sequence (cold MT; lane 3), Rb antibody (Rb-Ab; lane 4) or cyclin A antibody (CycA-Ab; lane 5). The E2F-DNA complexes are indicated. (b) Dose-response to *D-erythro*-sphingosine delivered in ethanol. Cells were treated with either ethanol vehicle or the indicated concentrations (μM) of *D-erythro*-sphingosine for 4 h; extracts were prepared and E2F-binding assay was performed. (c) Specificity of the effects of sphingosine. Cells were treated with ethanol vehicle (lane 1), 1 μM *D-erythro*-sphingosine (lane 2), 1 μM stearylamine (lane 3) or 1 μM dioctanoylglycerol (lane 4) for 4 h. Gels shown are representative of at least four different experiments.

E2F in complex with cyclin A as they were eliminated by addition of cyclin A antibody (Figure 5a, lane 5). These results are in agreement with previous results in U937 cells which also indicate that the Rb-related protein, p107, is an integral part of the E2F-cyclin A complex [37,38]. Whole-cell extracts from Molt-4 cells treated with increasing concentrations of *D-erythro*-sphingosine for 4 h showed gradual loss of all the specific complexes, with initial effects observed at a concentration of 0.6 μM and complete effects obtained with 1 μM (Figure 5b).

The specificity of this effect was examined by treating Molt-4 cells with various other lipids under identical conditions. Stearylamine and dioctanoylglycerol treatments did not affect E2F-DNA binding (Figure 5c).

To test the role of Rb in mediating this effect of sphingosine on E2F-DNA binding, we conducted the same experiment in WERI-Rb-1 cells, which lack functional Rb. As expected, growing WERI-Rb-1 cells lack the E2F-RB complex because of the absence of functional Rb. Instead, three specific complexes were detected that contain E2F-DNA binding activity (Figure 6a, lanes 1-3). The fastest complex was not affected by the addition of either Rb or cyclin A antibodies and represents free E2F (Figure 6a, lanes 4 and 5). This is further supported by incubating the cell extract with deoxycholate before assaying for E2F binding. The detergent effect of deoxycholate results in the loss of protein-protein interactions and conversion of the multiple complexes into a single DNA-protein complex typical of free E2F [33] (Figure 6a, lane 6). The slowest complex, E2F-cyclin A,

represents E2F in complex with cyclin A, as it was eliminated by the addition of cyclin A antibody (Figure 6a, lane 4). The intermediate complex, X, was not characterized further. Treatment of these cells with *D-erythro*-sphingosine at concentrations of 0.5-5 μM for 4 h had no effect on the E2F-DNA binding activity (Figure 6b). Therefore the presence of functional Rb is required to induce the specific loss of E2F-DNA binding activity observed after treatment with *D-erythro*-sphingosine at concentrations that induce potent Rb dephosphorylation. In addition, the loss of the E2F-cyclin A complexes observed in Molt-4 cells was not seen in WERI-Rb-1 cells. This indicates that sphingosine interacts specifically with Rb, but not the closely related protein p107.

In order to examine further the role of Rb in mediating the effects of sphingosine on E2F-DNA binding, similar experiments were carried out in the mink lung epithelial cell lines MK-13 and PVU-0 which differ only by their Rb status. Gel-shift experiments from Mk-13 cells show E2F-containing bands analogous to those present in Molt-4 cells except they are less well-resolved. The other difference is the presence of a substantial amount of free E2F which migrates slightly faster than the E2F-Rb complex (Figure 5c, first lane). In PVU-0 cells, the expression of wild-type large T antigen results in the disruption of E2F-Rb and E2F-p107-cyclinA complexes resulting in the appearance of a single band representing free E2F (Figure 6c, third lane). When MK-13 cells were treated with *D-erythro*-sphingosine at 30 μM for 6 h, a concentration sufficient to cause more than 50%

Figure 6 Effect of *D*-erythro-sphingosine on E2F-DNA binding in WERI-Rb-1, Mk-13 and PVU-0 cells

(a) Specific complexes in WERI-Rb-1 cells. Cells were extracted and gel-shift assays performed as in Figure 5. Lane 1, control sample incubated with labelled probe (labelled WT); lane 2, incubation in the presence of excess unlabelled E2F-binding oligonucleotide (cold WT); lane 3, incubation in the presence of excess unlabelled mutant oligonucleotide (cold MT); lane 4, incubation with cyclin A antibody (CycA-Ab); lane 5, incubation with Rb antibody (Rb-Ab); lane 6, cell extract preincubated in deoxycholate (DOC). (b) Dose-response to *D*-erythro-sphingosine. Cells were treated with ethanol vehicle or the indicated concentrations (μ M) of *D*-erythro-sphingosine for 4 h, and extracts and gel shift assays were performed as above. (c) Effect of *D*-erythro-sphingosine on E2F-DNA binding in mink lung epithelial cells. Mk-13 and PVU-0 cells were treated with 30 μ M *D*-erythro-sphingosine (30) or ethanol vehicle (0) for 6 h as indicated. Extracts and gel-shift assays were performed as above. Gels shown are representative of at least three different experiments.

growth inhibition, E2F-DNA binding was significantly inhibited (Figure 6c, second lane). When PVU-0 cells were treated with a similar concentration of sphingosine, the binding of E2F to its specific DNA sequence was not inhibited (Figure 6c, last two lanes). Therefore inhibition of E2F-DNA binding by sphingosine requires the presence of functional Rb. Furthermore, loss of E2F-DNA binding appears to correlate with growth inhibition induced by sphingosine.

D-erythro-Sphingosine down-regulates *c-myc* transcription

Next, we investigated the functional implications of the loss of E2F binding to its specific sequence element. E2F binding to its specific sites in the *c-myc* promoter has been shown to be important for *c-myc* transcription [24,39,40]. We studied the effects of *D*-erythro-sphingosine treatment of Molt-4 cells on the levels of *c-myc* mRNA levels detected by Northern blotting. Conditions identical with those described above for E2F-DNA-binding experiments were used except that treatments were for 6 h. There was a dose-dependent down-regulation of *c-myc* mRNA with no effect on control RNA. The effect was observed starting at a concentration of 0.6 μ M *D*-erythro-sphingosine (Figure 7a). This effect was specific for the *D*-erythro-sphingosine enantiomer, as *L*-erythro-sphingosine (see Figure 2 for structures) was unable to down-regulate *c-myc* mRNA (Figure 7b). Therefore *D*-erythro-sphingosine induced specific down-regulation of *c-myc* mRNA at concentrations similar to those that induced Rb dephosphorylation and loss of E2F-DNA binding activity.

DISCUSSION

Rb appears to be central to a pathway of cell regulation important in growth inhibition, tumour suppression and cell-cycle arrest. It exhibits its growth inhibitory effects when it is predominantly hypophosphorylated in G_0/G_1 and is inactivated by gradual phosphorylation as the cell cycle progresses only to become activated (dephosphorylated) again at the end of mitosis [21,22]. The physiological mechanisms responsible for regulating Rb are being explored. Several cyclins have been shown to result in Rb inactivation (phosphorylation) when overexpressed along with Rb in an Rb-deficient osteosarcoma cell line [30,31,41]. However, the physiological pathways responsible for Rb activation remain largely unknown. Our recent finding that sphingosine can potentially, specifically and rapidly result in Rb dephosphorylation provided the first opportunity to study the functional effects of activating this pathway, in this case by a lipid-breakdown product, and to define the mechanisms by which sphingosine inhibits growth.

In this study, we show that the potent and specific growth-inhibitory effects of sphingosine, but not the structurally related long-chain amino base stearylamine, are dependent on the presence of functional Rb. These effects are seen in the Rb-containing Molt-4 cells but not in WERI-Rb-1 cells which lack Rb. In addition, mink lung epithelial cells that have been transformed using either wild-type large T antigen of SV40 (PVU-0) or the K1 mutant which is unable to bind Rb (Mk-13) show a dramatically divergent response to sphingosine. Mk-13 cells containing functional Rb are very sensitive to the growth-

Figure 7 Effect of *D*-erythro-sphingosine on *c-myc* mRNA

(a) Dose-response to *D*-erythro-sphingosine. Molt-4 cells were treated with ethanol vehicle or the indicated concentrations of *D*-erythro-sphingosine for 6 h. Total RNA was isolated, electrophoresed, transferred to nitrocellulose membrane, and the blot was hybridized to a nick-translated 32 P-labelled *c-myc* cDNA fragment probe or GC-rich cDNA probe which strongly hybridizes to 28S RNA as indicated. (b) Specificity of the *D*-erythro-sphingosine on *c-myc* mRNA. Molt-4 cells were treated with ethanol vehicle (C), *D*-erythro-sphingosine (1 μM; D) or *L*-erythro-sphingosine (1 μM; L) for 6 h as indicated. *c-myc* mRNA was assayed as in (a). Blots shown are representative of four different experiments.

inhibitory effects of sphingosine, whereas PVU-0 cells, in which Rb is complexed by large T antigen, are almost completely resistant. A role for Rb-related proteins (especially p107) in mediating the susceptibility to sphingosine cannot be ruled out, although the resistance of WERI-Rb-1 cells, which contain p107 as evidenced by the specific gel-shift complexes, argues against a critical role. Therefore an Rb-dependent pathway of cell-growth inhibition is important in mediating the growth-inhibitory effects of sphingosine. Equally as important, these studies demonstrate that the effects of sphingosine on cell growth are not due to non-specific lipid effects, as cells that lack a downstream target for sphingosine (Rb) are significantly resistant to sphingosine-induced cytotoxicity.

Interestingly, we also show that higher concentrations of sphingosine and stearylamine result in equipotent growth inhibition of WERI-Rb-1 cells. Also, higher concentrations of stearylamine are capable of inhibiting Molt-4 cell growth (results not shown). We speculate that these effects are secondary to the ability of sphingosine, stearylamine and other amino bases to inhibit PKC [42] or possibly other targets that are equally sensitive to a variety of long-chain amino bases. Therefore sphingosine can potentially activate at least two distinct pathways of cell regulation. The first is specifically activated by *D*-erythro-sphingosine, resulting in dephosphorylation of Rb independent of PKC inhibition; the second is activated at higher concentrations of sphingosine and other amino bases, resulting in growth suppression. At a practical level, cells that lack a functional Rb would not exhibit the first (specific) pathway of growth inhibition.

In this study we also show that one of the downstream effects

of Rb dephosphorylation by sphingosine is the inhibition of E2F binding to its specific DNA motif. E2F binds to several specific and closely related DNA motifs in the promoters of several cellular genes important for cell proliferation. E2F can bind either in the free form, shown to be transcriptionally active, or in different complexes with other cellular proteins including Rb or p107, cyclin A and cdk2. In the current study, sphingosine inhibited the DNA binding of E2F and its complexes at concentrations similar to those capable of causing dephosphorylation of Rb and growth inhibition. These effects are also specific to sphingosine as neither stearylamine nor diacylglycerol was capable of producing similar inhibition at comparable concentrations. Furthermore, these effects required the presence of functional Rb as they were not seen in WERI-Rb-1 cells (compare Figures 5b and 6b) or in PVU-0 cells (Figure 6c). In published studies [43,44], cells that are growth arrested by serum starvation show similar loss of binding of E2F and its complexes to the specific DNA element. Therefore the effects of sphingosine treatment on E2F-DNA binding are analogous to those of serum starvation.

Factors that determine whether the E2F-Rb complex will bind to DNA or not are poorly understood. It is evident from this and other studies that a form of Rb can complex with E2F and still bind to DNA, perhaps as a transcriptionally negative element [36]. However, when affinity-purified E2F is added to Rb *in vitro*, no E2F-DNA binding activity is detected, although a less pure preparation exhibits significant DNA binding of a distinct Rb- and E2F-containing complex [45]. A 60 kDa protein, RBP60, was recently purified and appears to interact directly with Rb and to be necessary for imparting the DNA-binding ability of the E2F-Rb complex [45]. In contrast, Rb was found to be a functional component of E2F-I, an E1A-regulated inhibitor of the DNA-binding activity of E2F [46]. In other studies, unphosphorylated Rb was found to polymerize, forming a high-molecular-mass complex, and to be physically associated with a structural compartment in the nucleus [47-50]. Therefore a speculative model for E2F regulation would involve Rb in at least three forms that may have different levels of phosphorylation: hypo-, intermediate and hyper-phosphorylated. The hypophosphorylated form of Rb induced by sphingosine, serum deprivation or physiologically at the end of mitosis can polymerize and perhaps function as a 'corral' for E2F and other transcription factors [47,50]. In this form Rb may compete successfully for E2F binding with the Rb-related protein, p107, resulting in the sequestration of E2F and inhibition of its DNA-binding ability in multiple complexes. Intermediate phosphorylation of Rb, as would occur towards the end of G_1 , releases Rb from the high-molecular-mass complex while still bound to E2F to form a complex that is still capable of binding DNA in the presence of RBP60 [45]. Further phosphorylation of Rb results in an inactive monomer that is not associated with E2F.

In this study, we also look at an effect further downstream from E2F inhibition induced by sphingosine-activated Rb. The P2 promoter of the *c-myc* gene has been shown to contain two E2F-binding sites. Mutation of both these sites results in a significant decrease in basal transcription of *c-myc* [40]. Here we show that activation of Rb by sphingosine, which results in inhibition of E2F, is followed by downregulation of *c-myc*. Moreover, this effect is specific to the *D*-erythro enantiomer of sphingosine, the natural molecule.

It is unknown at this point whether sphingosine plays a physiological role in the regulation of Rb. The concentrations of sphingosine used in our experiments are within the reported range of cellular sphingosine levels [51]. The specificity of activity to the *D*-erythro-sphingosine enantiomer supports a role for

natural sphingosine or a closely related molecule. However, little is known about factors that regulate cellular levels of sphingosine and their effect on Rb dephosphorylation. Further experiments that are aimed at elucidating the physiological role of sphingosine in Rb regulation are in progress.

In conclusion, sphingosine can activate an Rb-dependent pathway of cell regulation, resulting in inhibition of the transcription factor E2F, subsequent down-regulation of *c-myc* mRNA transcription, and suppression of cell growth.

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