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The growth arrest and downregulation of c-*myc* transcription induced by ceramide are related events dependent on p21 induction, Rb underphosphorylation and E2F sequestering

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Abstract

Ceramide is an intracellular lipid mediator generated through the sphingomyelin cycle in response to several extracellular signals. Ceramide has been shown to induce growth inhibition, c-*myc* downmodulation and apoptosis. In this paper we examined the mechanism by which ceramide induces growth suppression and the role of the G1-CDK/pRb/E2F pathway in this process. The addition of exogenous, cell-permeable C_2-ceramide to the Hs 27 human diploid fibroblast cell line resulted in a dose-dependent induction of the $p21^{WAF1/CIP1/Sdi1}$ kinase inhibitor with reduction of cyclin-D1 associated kinase activity. Furthermore, significant dephosphorylation of pRb was observed, with increased association of pRb and the E2F transcription factor into a transcriptionally inactive complex. Ceramide was also capable of inhibiting the transcriptional activity of a CAT reporter vector driven by E2F binding sites containing c-*myc* promoter transfected into Hs 27 cells. The requirement of the pRb protein for ceramide-induced c-*myc* downregulation was supported by the failure of ceramide to inhibit promoter activity in HeLa cells, in which pRb function is abrogated by the presence of the E7 Papilloma virus oncoprotein, and in pRb-deleted SAOS2 AT cells. Ceramide-induced downregulation of the c-*myc* promoter was restored in SAOS2 #1 cells in which a functional Rb gene was reintroduced. Our studies demonstrate that pRb dephosphorylation, induced by ceramide, is at least partly necessary for c-*myc* down-regulation, and therefore the CDK-Rb-E2F pathway appears to be a target for the ceramide-induced modulation of cell cycle regulated gene transcription.

Keywords: ceramides, growth arrest, c-*myc*, $p21^{WAF1/CIP1/Sdi1}$

Abbreviations: TNFα, tumor necrosis factor α; pRb, Retinoblastoma gene product; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfaphenil)-2H-tetrazolium; C_2-ceramide, N-acetylsphingosine; DH-C_2 ceramide, dihydro-N-acetylsphingosine

Introduction

Sphingolipids constitute an important class of lipid molecules with important roles in tissue development, oncogenesis, cell growth, differentiation and apoptosis (Ballou, 1992a; Hannun, 1994; Kolesnick and Golde, 1994). In addition, sphingolipids have been reported to generate a senescent cellular phenotype in human cell lines (Venable *et al*, 1995).

Ceramide, a product of sphingomyelin hydrolysis, has recently emerged as a candidate intracellular mediator in response to several cellular stimuli such as TNFα (Dressler *et al*, 1992), IL1β (Mathias *et al*, 1993), γIFN (Kim *et al*, 1991), p75 LNGFR (Dobrowsky *et al*, 1994) and hydroxiD_3 (Okazaki *et al*, 1990) and it has been shown to mediate several biochemical activities, such as phosphorylation of the EGF receptor (Goldkorn *et al*, 1991), induction of cyclo-oxygenase (Ballou *et al*, 1992b), downregulation of c-*myc* (Wolff *et al*, 1994), modulation of the activity of protein kinases (CAPK) and phosphatases (CAPP) (Mathias *et al*, 1991; Dobrowsky and Hannun, 1992; Dobrowsky *et al*, 1993). Furthermore, ceramide-activated protein kinase has been shown to phosphorylate Raf on thr 269, increasing its activity upon MEK (Yao *et al*, 1995). Requirement for ceramide in signalling through SAPK/JNK has also been described in stress-induced apoptosis (Verheij *et al*, 1996). Finally, ceramide is a potential modulator of the nuclear kB activity (Yang *et al*, 1993), involved in the regulation of several cellular and viral genes.

At the cellular level ceramide has been shown to exert an antiproliferative effect, similar to the G0/G1 arrest observed in response to withdrawal of essential serum factors associated with suppression of c-*myc* expression. C-*myc* is one of the cell cycle regulated genes and is involved in cell proliferation, differentiation and apoptosis (Marcu *et al*, 1992; Packhman and Cleveland, 1995; Evan *et al*, 1992; Gu *et al*, 1994). In addition to growth arrest, ceramide is a strong inducer of apoptosis, and the prevalent effect depends on the doses and the cell types used in different experiments (Papp *et al*, 1994; Obeid *et al*, 1993).

Cell cycle control occurs mainly through the sequential activation of cyclin-dependent protein kinases (CDKs) which are programmed to phosphorylate regulatory proteins, thereby triggering events required for the G1-S transition. The decision to proceed beyond the late G1 appears to be executed by phosphorylating and thereby inactivating the product of the retinoblastoma gene (pRb),

whose growth-restraining role in early to mid-G1 reflects its ability to sequester a series of transcription factors the activities of which are required to induce expression of S-phase genes (Sherr *et al*, 1994; Lukas *et al*, 1996).

In fact, the molecular mechanism of Rb-dependent growth suppression is suggested to rely on physical interaction between the underphosphorylated Rb protein and the cellular transcription factor E2F. In fact, E2F plays a central role in the control of cell proliferation (Chellappan, 1994) and in the control of transcription of several growth-associated genes, including c-*myc*, N-myc, B-myb, cdc2, DHFR, thymidilate-synthetase, thymidine-kinase and DNA polymerase α genes, which contain E2F-binding sites in their regulatory regions (Blake and Azizkhan, 1989; Dalton, 1992; Helin and Harlow, 1993; Mudryj *et al*, 1990; Moran, 1993; Nevins, 1992; Pearson *et al*, 1991; Thalmeier *et al*, 1989; Vairo *et al*, 1995). Some of the latter genes have been shown to require E2F for their cell-cycle dependent expression while the transcriptional activity of E2F is inhibited by proteins of the retinoblastoma family, such as the retinoblastoma suppressor protein (pRb), p107 and p130 (Chellappan *et al*, 1991; La Thangue, 1994; Lees *et al*, 1992; Qin *et al*, 1992; Sardet *et al*, 1995). Inhibition of E2F activity is paralleled by cell growth suppression by retinoblastoma family members. The tumor suppression function of pRb also appears to correlate with its ability to repress E2F-dependent transcription. Naturally occurring mutants, which have lost pRb function, as well as the wild type pRb, which interacts with viral oncogenic proteins like E1A of Adenovirus or E7 of Papilloma virus, are unable to regulate E2F (Arroyo and Raychaudhuri, 1992; Hiebert, 1993). In the physiological cellular context, the growth inhibitory properties of pRb are eliminated by CDK-dependent phosphorylation of the protein (Chellappan *et al*, 1991; DeCaprio *et al*, 1989; Dynlacht *et al*, 1994; Hunter and Pines, 1991) that subvert its ability to bind to and inactivate E2F. Recently, a family of CDK inhibitors has been identified. Well characterized members of this family are p21, p27 and p57. The cDNA of p21 was cloned by virtue of encoding an overexpressed transcript in senescent human fibroblasts (Noda *et al*, 1994), a p53-inducible transcript (El-Deiry *et al*, 1993) and a Cdk2-associated protein (Xiong *et al*, 1993). p21 inhibits the activity of CDK-cyclin complexes and the onset of DNA replication. p21 probably blocks DNA replication, at least in part, by inhibiting pRb phosphorylation, followed by inhibition of E2F activation in late G1.

Although ceramide has been reported to induce Rb underphosphorylation (Dbaibo *et al*, 1995) and to down-regulate cell growth, as well as cell cycle regulated genes (e.g. c-*myc*), there is only marginal information about the biochemical and molecular pathways involved. This prompted us to study whether c-*myc* regulation might be linked to the phosphorylation status of Rb and whether the effect of ceramide might be mediated by modulation of the cellular levels of the kinase inhibitors p21 and p27. Our results suggest that the observed ceramide-induced c-*myc* downregulation is likely to be generated at the transcriptional level through a mechanism involving different steps: (i) up-regulation of the p21 kinase inhibitor; (ii) reduction of cyclin D1-regulated kinase activities; (iii) Rb underphosphorylation; (iv) reduced availability of transcriptionally functional E2F for the two E2F sites located in the c-*myc* regulatory region with subsequent decrease of c-*myc* transcription. Therefore, the CDK-Rb-E2F pathway appears to be a target for the ceramide induced regulation of cell cycle regulated gene transcription.

Results

Ceramide induces growth arrest and apoptosis in Hs 27 human fibroblasts

In order to analyze the biochemical pathway through which ceramide induces its antiproliferative or apoptotic activity, we used young Hs 27 human diploid fibroblasts (20–30 passages) growing rapidly as shown by the (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium, inner salt (MTS) assay. The cell cultures were analyzed for apoptosis, one of the major biological effects of ceramide (Figure 1A). The number of apoptotic fibroblasts increased progressively with the dose of ceramide added to the cultures and with the time of treatment; up to 26% of apoptotic cells were observed at 48 h with 30 μM C_2-ceramide as analyzed by microscopic count after AO/EtBr staining. In control cells treated with 30 μM DH-C_2 ceramide, no apoptosis was detected at the same time intervals. DH-C_2 ceramide is a biologically inactive homologue of C_2-ceramide which lacks the trans-double bond at C_4-C_5 of the sphyngoid base backbone. Remarkedly, treatment of Hs 27 with cell-permeable C_2-ceramide (5 and 30 μM) also induced a dose-dependent inhibition of cell proliferation, whereas no replicative arrest was observed after DH-C_2 ceramide treatment (Figure 1B).

Effect of ceramide on CKI levels and on CDKs activities

Whole cell extracts from the above culture were subjected to SDS–PAGE, followed by electrotransfer onto nitrocellulose, and the level of the different proteins involved in cell cycle regulation were analyzed.

The major finding of our experiments is shown in Figure 2, where crude extracts from control (DH-C_2 ceramide 30 μM) or treated cells (C_2-ceramide 5 and 30 μM) were analyzed for p27 and p21 levels after an 8 h exposure to the drug. This experiment demonstrated that whereas a slight decrease in p27 (Figure 2B) was induced by ceramide, a significant increase of p21 was observed in the same sample (Figure 2A). The p21 increase may represent the biochemical connection between ceramide treatment and proliferative arrest, since p27 and p21 kinase inhibitory action converge together to generate the G1-inhibitory outcome of kinase regulation. Therefore, we evaluated the kinase activity (Figure 3) in immunoprecipitates with anti-cyclin D1 MAb by an *in vitro* kinase assay. The D1-associated kinase activity was strongly reduced after ceramide treatment when assayed either on histone-H1 (Figure 3A) or on the physiological substrate recombinant pRb (Figure 3B). When the same immunoprecipitates were subjected to analysis for the presence of p21 in the complexes, an increased amount of this kinase

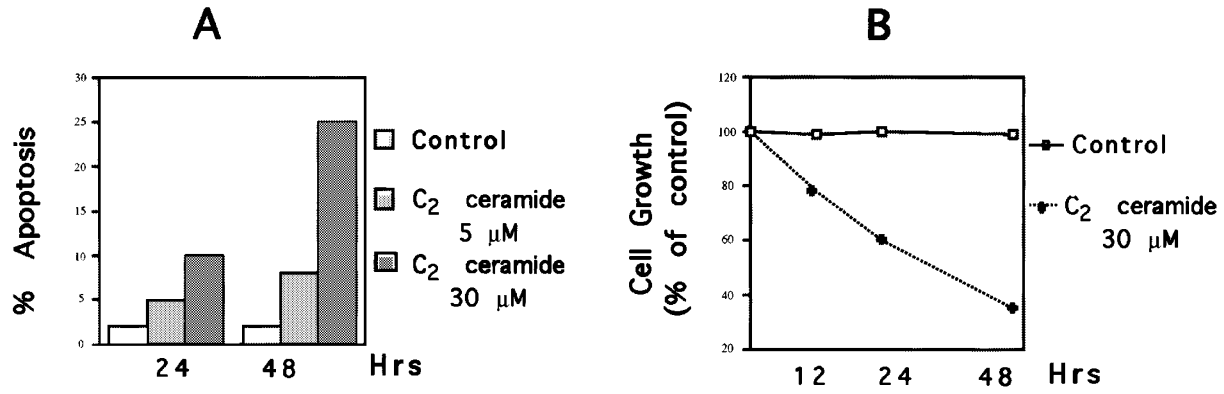

Figure 1 Effect of C_2-ceramide on apoptosis and growth of Hs 27 human diploid fibroblast. Growing Hs 27 at about 22 PD were treated with DH-C_2 ceramide (30 μM as control) or C_2-ceramide (5 or 30 μM) and analyzed for apoptosis and cell growth after 24 and 48 h. (**A**) the evaluation of apoptosis was performed on living cells using AO/Etbr (100 μg/ml each) and fluorescence microscopy. For each determination 400 cells were examinated (DH-C_2 ceramide 30 μM, C_2-ceramide 5 μM, C_2-ceramide 30 μM) and the experiment was performed in triplicate. In one separate experiment similar data were confirmed by cytofluorimetric analysis. (**B**) a colorimetric (MTS) assay was used to evaluate the cell proliferation over time. The data showed are representative of three different experiments. The difference observed either on proliferation and apoptosis at 24 and 48 h between treated and control were statistically significant ($P<0.01$)

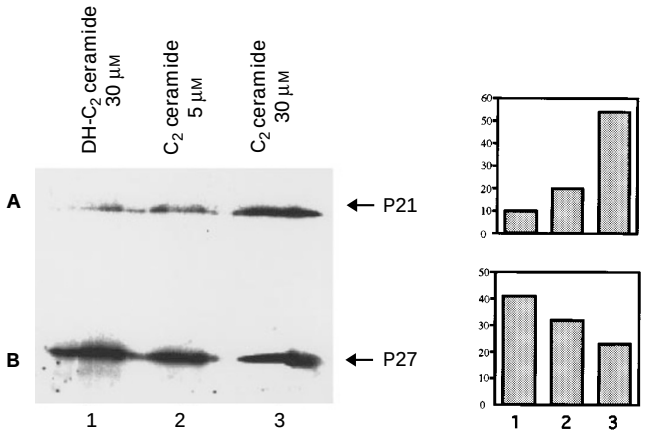

Figure 2 Western blot analysis for the peptide reactive with anti-p21 (**A**) and anti-p27 (**B**) monoclonal antibodies in total cell extracts from Hs 27 diploid fibroblasts treated with DH-C_2 ceramide (30 μM, lane 1), or with C_2-ceramide (5 μM, lane 2 or 30 μM, lane 3). Actively proliferating Hs 27 cells were treated with ceramides in medium containing 10% fetal calf serum. After 8 h cells were lysed and the protein extract analyzed in immunoblot. Increased levels of $p21^{WAF1,CIP,\ Sdi1}$ (**A**) and a small decrease of p27 kinase inhibitor (**B**) were detected in three separate experiments. The right half of each panel represents the densitometric analysis of the bands resulting from ECL reaction after autoradiography. Statistical analysis comparing the optical density of the band was performed and the p21 increase was significant ($P<0.01$). By contrast the p27 decrease was not statistically significant

inhibitor associated with cyclin D1 was detectable after ceramide treatment compared to the control (Figure 3C). This result may indicate that in rapidly growing fibroblasts, treated with C_2-ceramide, the level of p21 associated with cyclin D1 strictly determines the cyclin D1-dependent kinase activity.

Ceramide determines Rb underphosphorylation

Considering that pRb is one of the most relevant targets for the G1 kinases, we evaluated the phosphorylation status of this tumor suppressor protein, in Western blot analysis, by using whole cell extracts from the control or ceramide-treated Hs 27 cells. As shown in Figure 4, the treatment with DH-C_2 ceramide (30 μM, lane 1) was ineffective in inducing Rb modification, whereas a progressive loss of phosphate groups was apparent at increasing C_2-ceramide concentrations (5 and 30 μM), as indicated by the shift in the mobility of the pRb MAb immunoreactive bands (Figure 4 lanes 2 and 3).

Effect of ceramide on pRb-E2F complexes and E2F DNA binding activity

Underphosphorylated pRb has been reported to form specific complexes with polypeptide partners, including E2F. The result of this interaction is the functional inactivation of the transacting factor with concomitant modification of the E2F DNA binding activity. To evaluate whether ceramide-induced underphosphorylation of Rb was able to induce the formation of Rb-E2F complexes and to influence E2F binding to a specific DNA consensus sequence, we tried two different approaches. First, we tested the levels of E2F-pRb complexes in control (DH-C_2 ceramide, 30 μM) and C_2-ceramide (30 μM) treated cells. Figure 5B shows that ceramide treatment of Hs 27 cells resulted in slightly increased levels of E2F-1 co-immunoprecipitated with pRb. The increase in the amount of E2F associated to pRb was not a consequence of higher levels of cellular E2F-1 in ceramide treated cells *vs* untreated cells, as a slight decrease in the total amount of E2F-1 was observed after treatment (Figure 5A). These data suggest that ceramide is capable of increasing the levels of E2F-pRb complexes, even though the total levels of E2F1 are, *per se*, reduced. To study whether ceramide-stimulated E2F-pRb complex formation could modify E2F binding activity, we then tested whole cell extracts from DH-C_2 ceramide-treated or C_2-ceramide-treated cells for the presence of the E2F DNA consensus sequence complexes (Figure 5C) using electromobility shift assay (EMSA). By using the E2F consensus sequence (-70

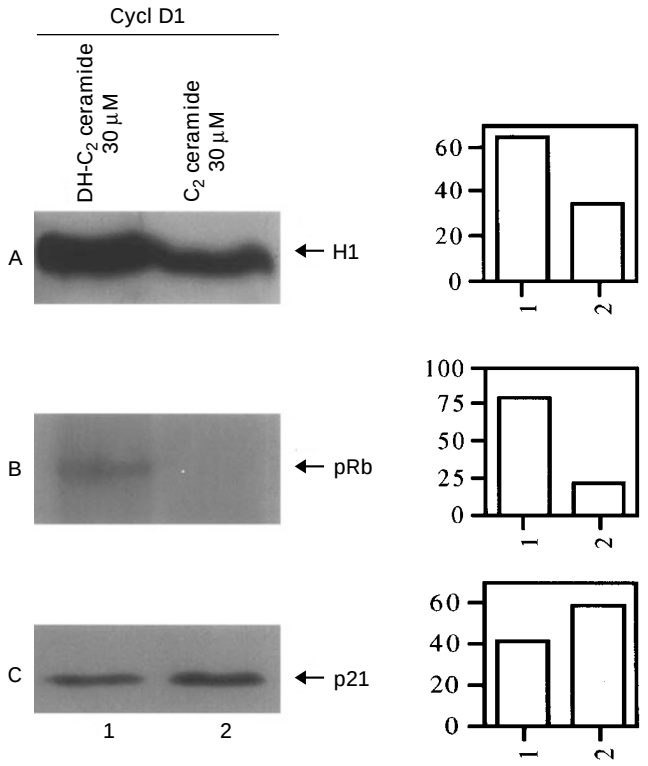

Figure 3 (**A**) *In vitro* kinase assay experiment to evaluate the kinase's activity associated with D1 cyclin. H1 histone was used as substrate for cellular kinase. Hs 27 were treated for 8 h with DH-C_2 ceramide 30 μM (lane 1) or with C_2-ceramide 30 μM (lane 2). The cells were lysed and the proteins were immunoprecipitated with monoclonal antibody to cyclin D1. The cyclin associated kinases present in the immunoprecipitates were evaluated for their activity by the artificial phosphorylation of the H1 histone using the phosphate group of γ^{32}P-ATP. (**B**) *In vitro* kinase assay to evaluate the kinase's activity associated with D1 cyclin on recombinant pRb. (**C**) Western blot analysis was performed with a p21 MAb to detect the immunoreactive peptides present in the D1 cyclin immunoprecipitate complexes after DH-C_2 ceramide or C_2-ceramide treatment (30 μM, 8 h). The right half of each panel represents the densitometric analysis of the autoradiograms. Similar results were observed in three different experiments. Whereas the result of **A** and **B** were statistically highly significant (optical density of DH-C_2 ceramide *versus* optical density of C_2-ceramide, $P<0.01$) the result of (**C**), even if consistently repeated, did not reach statistical significance

to −30 bp) of the c-*myc* promoter, we observed a decrease in the E2F-DNA complexes after ceramide treatment compared to the control, probably due to the reduced E2F-1 levels. On the other hand, we were unable to detect an increase in the supershifted E2F-pRb complex after ceramide treatment, probably because of the low amount of residual E2F-1 protein. This suggests that ceramide impairs the transcription factor's ability to bind and transactivate the E2F cognate binding site of the c-*myc* promoter, either by reducing the levels of E2F1 or by inducing E2F association to pRB.

Ceramide downregulates the transcriptional activity of the c-*myc* promoter: requirement for pRb

To investigate whether the ceramide-induced Rb underphosphorylation, the reduction in the E2F1 levels, or both,

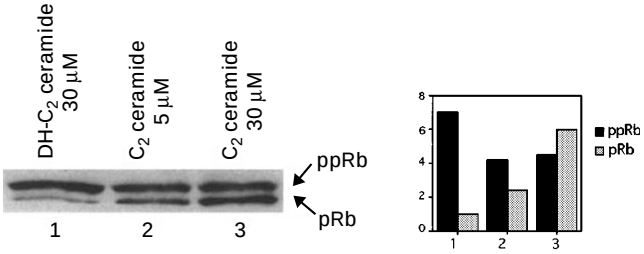

Figure 4 Rb phosphorylation status in whole cell extract from Hs 27 at about 22th passage. Western blot analysis was performed with anti-Rb MAb on crude extracts from control cells (treated with DH-C_2 ceramide 30 μM, lane 1), or from cells treated with 5 μM (lane 2) or 30 μM (lane 3) of C_2-ceramide for 8 h. The proteins were resolved on SDS–PAGE (8.5%), transferred onto nitrocellulose and after immunoreaction resolved by ECL. The position of hypophosphorylated and hyperphosphorylated forms of pRb are indicated. A shift from the Rb phosphorylated form (lane 1) to underphosphorylated form was evident and dose dependent (lanes 2 and 3). Each lane was loaded with 50 μg of total proteins and the amounts of protein loaded was confirmed by Ponceau S staining. Three different experiments confirmed this result. The right half of the figure is the schematic representation of the optical density of the bands. The black bars represent hyperphosphorylated pRb and the white bars the hypophosphorylated form

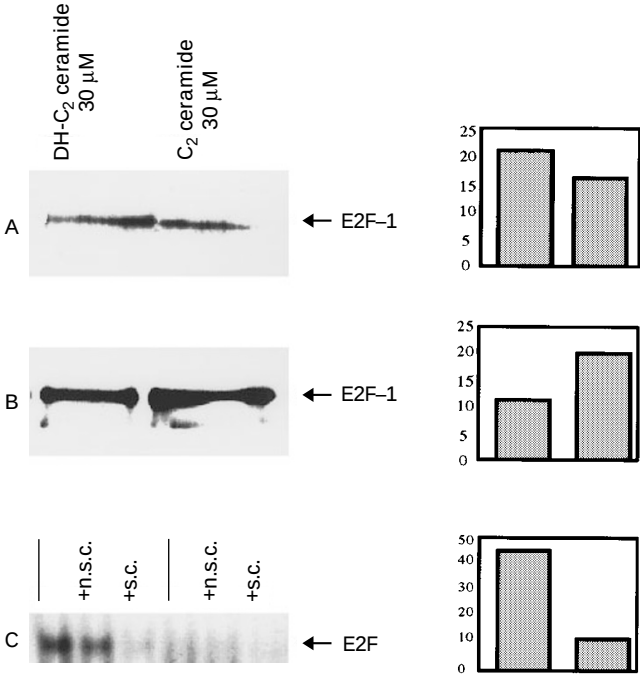

Figure 5 (**A**) Western blot analysis for E2F-1 levels in crude extract from Hs 27 cells treated with DH-C_2 ceramide (30 μM) or C_2-ceramide (30 μM) for 8 h. (**B**) Hs 27 crude extracts from cells treated with DH-C_2 ceramide or C_2-ceramide as above were immunoprecipitated with anti-Rb MAb, resolved on SDS–PAGE, electrophoretically transferred onto nitrocellulose and analyzed for the amount of E2F-1 coimmunoprecipitated with pRb using anti-E2F-1 MAb. (**C**) E2F complexes in Hs 27 whole cell extracts treated with DH-C_2 ceramide or C_2-ceramide (30 μM) detected by electromobility shift assay (EMSA). The probe used was an oligo matching the E2F site in the c-*myc* promoter. The specificity of the bands was determined by competition with either cold E2F-*myc* promoter site (specific competitor, s.c.) or E2F c-*myc* promoter mutated site (non specific competitor, n.s.c.). The right half of each panel represent the densitometric analysis of the specific bands (**A**, **B** and **C**, respectively) after autoradiography of one of three repeated experiments. The statistical analysis by comparing the optical density of the bands was performed (**A**: $P<0.05$, **B** and **C**: $P<0.01$)

correlated with the ceramide effect on c-*myc* gene transcription, we transfected Hs 27 cells with a reporter gene containing 250 bp of the c-*myc* regulatory region including the P1 and the P2 promoters and the E2F sites upstream of the CAT coding sequence.

As shown in Figure 6, a significant reduction of c-*myc* promoter-driven CAT expression was observed in the Hs 27 cell line after 8 h of ceramide treatment (spots above lane 2 *versus* spots above lane 1), whereas no effect was detectable in HeLa transfected cells, harboring the oncogenic E6 and E7 proteins of the Papillomavirus, the latter being involved in Rb inactivation. Failure of ceramide to downregulate the c-*myc* promoter in HeLa cells suggests that sequestering of pRb by E7 proteins might be, at least partly, responsible for the lack of this effect in addition to E2F reduction. Therefore, to study whether pRb is required for ceramide-induced downregulation of the c-*myc* promoter, we transfected a c-*myc* promoter-CAT vector into the SAOS2 AT cell line harbouring a functionally significant Rb deletion, and into the SAOS2 #1 cell line in which the full length gene was reintroduced, in the presence of either C_2-ceramide or DH-C_2 ceramide. Figure 7A shows that ceramide decreases the activity of the c-*myc* promoter in SAOS2 #1 (first couple of spots *versus* second couple of spots), whereas no effect on gene expression is observed in SAOS2 AT (third couple *versus* fourth couple of spots). Figure 7C represents the Western blot analysis of pRb in SAOS2 #1 and SAOS2 AT extracts. In addition to the endogenously deleted p95 pRb, the SAOS2 #1 cell line also expresses low levels of the exogenous p105 Rb. Remarkedly, HeLa and SAOS2 AT cells were resistant to growth suppression and cell cycle arrest observed after ceramide treatment in cells harbouring functional Rb.

Our observations suggest that the effect of ceramide requires the presence of a functional pRb which, by interacting with E2F, prevents the transcription factor from transactivating the c-*myc* promoter.

Discussion

Recent studies have demonstrated that withdrawal of serum factors from cultures of different cell types induces growth arrest (Marcu *et al*, 1992) associated with a significant increase in ceramide content (Jayadev *et al*, 1995). This finding strongly suggests that ceramide plays a role in mediating the growth suppressive effects of serum deprivation. At the same time, it is well known that starvation, as well as ceramide, induces downregulation of the c-*myc* oncogene (Wolff *et al*, 1994; Packhman and Cleveland, 1995). The role of this oncogene in regulating cell proliferation, differentiation and other important functions has recently been well established (Marcu *et al*, 1992; Packhman and Cleveland, 1995; Evan *et al*, 1992; Gu *et al*, 1994).

In this paper we analyze the biochemical steps leading to c-*myc* downregulation following ceramide treatment, with its obvious consequences in cell cycle progression. An interesting approach to study the antiproliferative properties of ceramide is to investigate whether its administration to cells modifies the levels, or the function, of cell cycle controlling proteins. At first, we analyzed the levels of p21 and p27 to comprehend the subsequent modulation of the G1 cyclin-dependent kinase activities. Since pRb is the most important protein substrate for CDKs phosphorylation during G1-S transition, we also analyzed the phosphorylation status of this protein. The major finding of our investigations is the dose-dependent increase in p21 levels induced by ceramide. With respect to the mechanism of p21 induction during ceramide treatment, we are

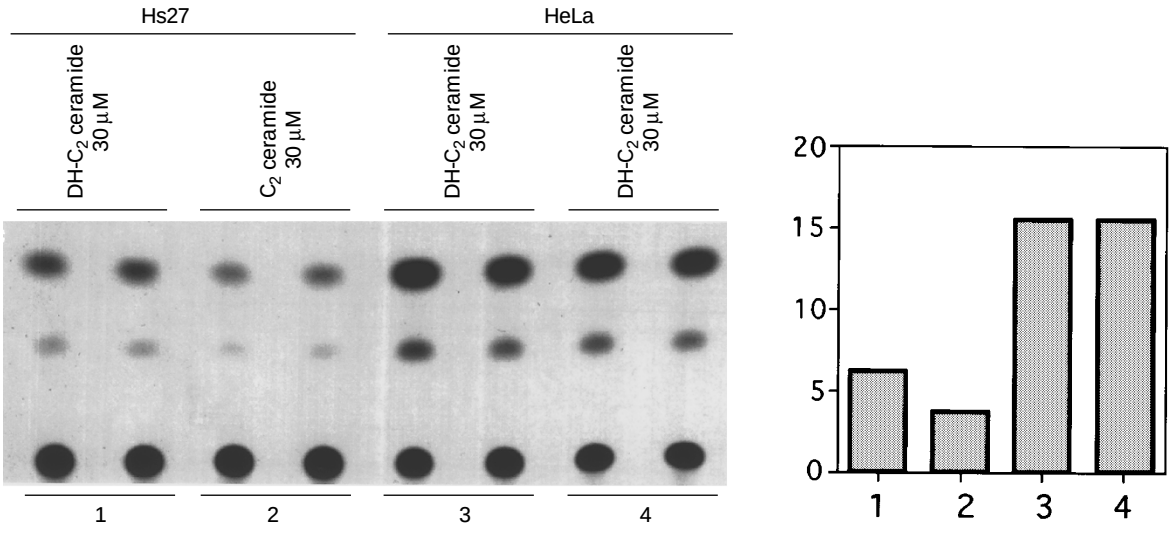

Figure 6 Transfection experiment using two different cell lines: Hs 27 (1–2) and HeLa (3–4). A *myc* CAT reporter gene, containing a 250 bp region encompassing the P1 and the P2 promoter of the gene, was introduced by DOTAP into the cells. After 48 h, the medium was replaced and DH-C_2 ceramide (1 and 3) or C_2-ceramide (2 and 4) were added at the final concentration of 30 μM. Each treatment was performed in duplicate. After further 8 h, the cells were harvested and proteins extracted by conventional protocol. The level of CAT activity was determined by the acetylation of [^{14}C]chloroamphenicol. All cells were cotransfected with pCH 110 as internal control for transfection efficiency. CAT activities were quantified by autoradiography followed by scanning radioactive acetylated spots (right half of the figure). The experiment was repeated three times and the difference in CAT expression after C_2-ceramide treatment of Hs 27 cells was statistically significant ($P < 0.01$)

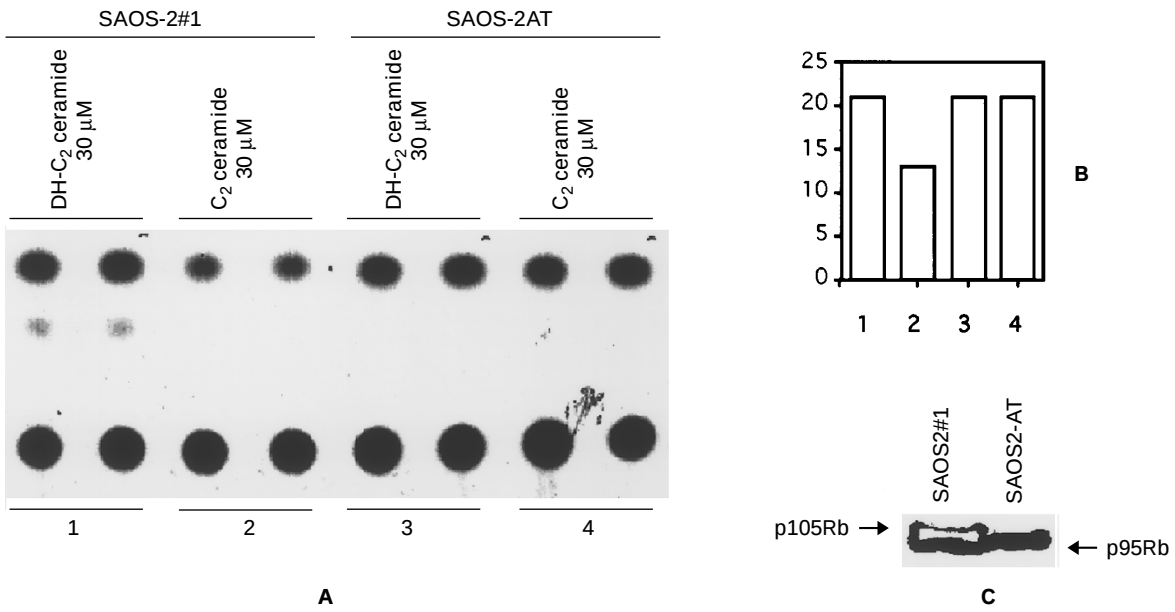

Figure 7 (**A**) Transfection of SAOS2 #1 (1–2) in which a functional Rb gene has been reintroduced, and SAOS2 AT (3–4), a pRb deleted osteosarcoma cell line, with c-*myc*-CAT plasmid. 10 μg of construct were introduced by DOTAP into subconfluent cultures. After 48 h, the medium was replaced and DH-C_2 ceramide (1 and 3) or C_2-ceramide (2 and 4) were added (final concentration 30 μM). Each treatment was performed in duplicate. After 8 h the cells were harvested and proteins extracted by a conventional protocol. CAT activities were quantified by autoradiography followed by scanning radioactive acetylated spots (**B**). A plasmid (pCH 110) was cotransfected to monitor transfection efficiency. Two different experiments were performed obtaining similar results and the difference between the optical density of couple 1 *versus* couple 2 was statistically significant. (**C**) Protein extracts prepared from parented SAOS2 AT cell line and from SAOS2 #1 were analzyed by Western blot for pRb expression. The partially deleted endogenous p95Rb and the exogenous p105Rb are indicated

actually analyzing the effect of ceramide at the transcriptional level of p21 regulation. Nevertheless, since ceramide is a pleiotropic molecule able to modulate several biochemical pathways, other mechanisms to explain p21 upregulation cannot be excluded. p21 upregulation might, *per se*, unequivically explain the cascade of molecular and biological events observed after ceramide treatment. In fact, p21 is a potent inhibitor of CDKs activity and cell proliferation. Furthermore, the inhibition of cyclin D1 dependent kinase by ceramide correlates with Rb underphosphorylation and E2F association into an inactive transactivation pRb-E2F complex. In addition, recent data suggest that the inhibition of cyclin-dependent kinases by p21 is an upstream event that downregulates the expression of E2F1 and of E2F-dependent transcription (Good *et al*, 1996). Consistent with our results, a recent report showed that p21 inhibits E2F-dependent transcription of several genes involved in cell cycle progression (Shiyanov *et al*, 1996). The regulatory region of the c-*myc* proto-oncogene contains two E2F binding sites, between the two major promoters, which are regulated during cell cycle progression in response to proliferate siganls (Pearson *et al*, 1991), and which are down-modulated by growth factor deprivation (Marcu *et al*, 1992) also resulting in a rise in intracellular ceramide (Jayadev *et al*, 1995). Therefore, we chose this gene as a model to investigate ceramide effects on the transcription of genes involved in cell cycle progression. We demonstrated that ceramide reduces binding of E2F to the cognate binding site located in the c-*myc* promoter and reduces its transcriptional activity only in cells harbouring a functional Rb protein (Hs 27, SAOS2 #1). On the contrary, this effect was not observed in cells lacking pRb (HeLa and SAOS2 AT) in which the pRb-E2F pathway is inactivated. On the basis of our results, we believe that pRb inactivation of E2F, in addition to the reduction of E2F1, is responsible for the observed c-*myc* downregulation.

In conclusion, the p21 modulated cyclin-dependent kinase-pRb-E2F pathway appears to be a target for the ceramide-induced regulation of cell cycle dependent genes. We also suggest that c-*myc* downmodulation induced by ceramide might represent a general model for gene regulation shared by all genes containing E2F sites in their regulatory regions. Since both pRb and E2F are members of gene families, with partially differentiated function, it would also be interesting to evaluate the effects of ceramide treatment on the function of other members of these families.

Materials and Methods

Cell culture and proliferation

Hs 27 human diploid fibroblasts and HeLa cells were obtained from ATCC (Gaithersburg, MD) and the Hs 27 were used in our experiment between passage 22 and 28. The cells were grown in Dulbecco's modified essential medium with the addition of 10% FCS, glutammine (2 mM) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin; Hyclone, Europe) at 37°C in a humidified incubator under 5% CO_2. The SAOS2 AT osteosarcoma cell line, which carries a naturally

occurring Rb gene deletion (exons 21–27) and the SAOS2 #1, a Rb reconstituted clone already described (Lukas *et al*, 1994; Fung *et al*, 1993), were grown in RPMI 1640 medium without or with geneticin (50 ng/ml) respectively.

Cell proliferation was measured in Hs 27 cells grown on a 96 well flat bottom microtiter plate using the MTS colorimetric assay (Cell Titer 96^{TM} AQeous Non Radioactive Cell Proliferating Assay from Promega, Madison, WI) (Buttke *et al*, 1993).

Morphological measurement of apoptosis

Confluent cultures were assayed for apoptosis and necrosis by fluorescence microscopy following labeling with acridine orange and ethidium bromide, as described by Duke and Cohen (1992). Floating and attached cells were collected as described above and washed three times in PBS. One-ml aliquots of 1×10^6 cells were centrifuged ($300 \times g$), the pellet was resuspended in 25 μl of media, and 1 μl of a dye mixture containing 100 μg/ml acridine orange and 100 μg/ml ethidium bromide prepared in PBS, and mixed gently. Ten ml of mixture were placed on a microscope slide and covered with a 22 mm^2 coverslip and examined under $\times 40$ dry objectives using epillumination and filter combination. An observer blinded to the identity of treatments scored at least 200 cells/sample. Live cells were determined by the exclusion of ethidium bromide stain. At least 400 cells were counted from each condition (control or C_2-ceramide treated cells) and apoptotic scoring was independently determinated by two observers. Each experiment was repeated three times.

Immunoprecipitation

Whole cell extracts (100–200 μg) were incubated with anti-pRb monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 4°C in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, 0.5 mM DTT and 3 mg/ml BSA. The mixtures were then further incubated with protein A/G plus Agarose (Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at 4°C. The beads were centrifuged and washed four times with 0.8 ml of the same buffer. The pellets were resuspended in loading buffer, resolved on 8.5% SDS–PAGE, transferred onto nitrocellulose and the filter was then used for Western analysis using anti-E2F1 monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany).

Western blot analysis

Cells treated with either C_2-ceramide or DH-C_2 ceramide (Biomol, Plymouth Meeting, PA) for 8 h were washed twice with ice-cold phosphate buffer saline, scraped off plates into hypotonyc lysis buffer (20 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium orthophosphate, 0.25 mM EDTA, 0.5 mM PMSF, 10 μg/ml leupeptine and 1% aprotinin) and then flash frozen in liquid nitrogen. After three cycles of freeze-thaw, the cells were passed several times through a 25 G needle. Lysates were cleared by centrifugation at $15\,000 \times g$ for 30 min, and protein concentrations were determined using the Biorad protein assay reagent (Biorad). Equal amounts of protein (usually 30 μg) were separated by SDS–PAGE, electrophoretically transferred onto nitrocellulose (Schleicher and Schuell, Germany) and probed with mouse monoclonal anti-Rb, anti-p21, anti-p27, or anti-E2F1 (Santa Cruz Biotechnology, Heidelberg, Germany). Immunoreactive bands were visualized by enhanced chemioluminescence (ECL, Amersham, UK). The films were then subjected to densitometric analysis for quantification of band densities. In some experiments, we used the same filter for both the *in vitro* kinase assay of H1 histone and Western blot in order to detect the p21-D1 cyclin immunoprecipitated complexes (Santa Cruz Biotechnology, Heidelberg, Germany).

In vitro kinase assay

This assay was performed according to Harper (Harper *et al*, 1993). Briefly, whole cell extracts from Hs 27 cells (10 centimetres subconfluent dish) treated with C_2-ceramide or DH-C_2 ceramide (20 μM) were obtained by lysis in 0.8 ml RIPA buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS and protease and phosphatase inhibitors). Each half of the extract was immunoprecipitated with cyclin D1 monoclonal antibodies (Santa Cruz Biotechnology, Heidelberg, Germany), and used for histone H1 (Sigma) or recombinant pRb (Santa Cruz Biotechnology) kinase assay in a 25 μl reaction containing 65 mM k-β-glycerophosphate (pH 7.3), 15 mM $MgCl_2$, 15 mM EGTA, 10 mM DTT, 1 mg/ml ovalbumin, 0.5 mM NaF, 0.1 mM sodium orthovanadate, 5 μg/ml leupeptin and 0.4 nCi/nmol [γ^{32}]ATP. Each reaction contained 2.5 μg of histone H1 or 5 μg pRb and a quarter of the immunoprecipitates with cyclin D1. After 15 min at 37°C, 5 μl aliquots were removed and spotted on phosphocellulose paper for quantification. The remainder of the aliquots were mixed with 15 μl of $2 \times$ SDS buffer prior to SDS–PAGE and autoradiography.

Cell transfections

A myc-CAT construct containing two potential E2F binding sites was produced by placing a *Not*I–*Mro*I fragment from the human c-*myc* regulatory region (250 bp) upstream of the CAT coding region of a conventional reporter gene (Basic CAT, Promega) after subcloning into the superpolylinker of the psL1180 plasmid (Pharmacia Biothec, Uppsala, Sweden). Hs 27, HeLa, SAOS2 AT and SAOS #1 cells were grown to 70% confluency and transfected by DOTAP reagent (Boehringer Mannheim) with 10 μg of myc-CAT construct. Forty-eight hours after transfection, fresh medium and DH-C_2 ceramide (30 μM) or C_2-ceramide (30 μM) was added. Twelve hours later, the cells were washed with ice-cold PBS and scraped-off the plates. The pellets were resuspended in 100 μl of Tris 0.25 M (pH 8.0) and stored at -80°C until used. Chloramphenicol acetyltransferase (CAT) assay was performed as described previously (Gorman *et al*, 1982). A pCH 110 plasmid (Pharmacia) was cotransfected to measure efficiency. CAT activities were quantified by autoradiography followed by scanning radioactive acetylated spots through phosphoimager.

Extracts preparation and EMSA

Whole cell extracts were prepared as described by Mudryj *et al* (1991), with some modifications. In short, cells were washed twice with cold phosphate buffered saline (PBS), scraped, and the pellet of approximately 5×10^7 was resuspended in 100 μl of cell lysis buffer (10 mM HEPES pH 7.6, 50 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF, 0.5 mM $NaVO_4$) and incubated in ice for 15 min. The cells were homogenized in a Dounce (30 strokes) and then 100 μl of extraction buffer (20 mM HEPES pH 7.6, 1.6 M NaCl, 20% glycerol, 0.1 mM EDTA, 0.5 NaF, 0.5 mM $NaVO_4$, 1 mM DTT) were added. The extracts were rocked for 30 min at 4°C, and then centrifuged for 60 min in 50 a Ti rotor at 38 000 r.p.m. The supernatants were removed and dialyzed at 4°C by 50 mM NaCl. For E2F, an assay band shift was performed as already described (Mudryj *et al*, 1991). The probe was an E2F oligonucleotide (0.2 ng/reaction) representing the distal E2F binding site of the c-*myc*

promoter (−70 to −30) with the following sequence: 5′-CGCTTGGCGGGAAAAAGAACGGAGGGAGGGATCGCGCTGAG. The mutated E2f site used for competition had the following sequence: 5′-GCTTGGATTGTTCAAGAACGGAGGGAGGGTACTGCTTGAG. 5 μg of crude extract were preincubated for 10 min at room temperature in the binding reaction containing 20 mM HEPES, 1 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 0.5 mM DTT, 100 ng poly(dI-dC), and 50 μg BSA. The ^{32}P-labeled probe was then added and the incubation continued for 20 min. A mutant E2F oligo was used to test binding specificity. The DNA-protein complexes were resolved on a 5% polyacrylamide gel in 0.25 × Tris-borate-EDTA (TBE) buffer, dried and autoradiographed.

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