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# Ceramide regulates SR protein phosphorylation during adenoviral infection

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**Abstract**

In this study, we show that adenoviral infection induced accumulation of the sphingolipid ceramide in a dose- and time-dependent manner. This accumulation preceded cell lysis, occurred in the absence of biochemical evidence of apoptosis, and was derived from de novo synthesis of ceramide. An adenovirus mutant that lacks the adenovirus death protein (ADP) produced ceramide accumulation in the absence of cell lysis. This suggested that ceramide accumulation was either driven by adenovirus or was a cellular stress response but was unlikely a result of cell death. The use of inhibitors of ceramide synthesis resulted in a significant delay in cell lysis, suggesting that ceramide was necessary for the lytic phase of the infection. Serine/arginine-rich (SR) proteins were dephosphorylated during the late phase of the viral cycle, and inhibitors of ceramide synthesis reversed this. These findings suggest that adenovirus utilizes the ceramide pathway to regulate SR proteins during infection.


*Keywords:* Adenovirus; Ceramide; SR proteins; Lipid mediators; Cell lysis

## Introduction

Adenoviruses are among the most common viral infections affecting human beings of all age groups (Kojaoghlanian et al., 2003; Walls et al., 2003). In the past two decades, remarkable advances in our understanding of their cycle and their clinical importance have been made. The study of adenoviruses has contributed to numerous discoveries that improved our understanding of viral and cellular gene expression and regulation, DNA replication, cell cycle control, and cellular growth regulation (Nevins, 1981, 1995). More recently, adenoviruses are being used as novel therapeutic tools, both as vectors of gene therapy and as oncolytic components of multimodal cancer therapy (Doronin et al., 2001; Nemunaitis et al., 2000; Post et al., 2003; St George, 2003).

Adenovirus contains several genes that usurp cellular signaling pathways involved in the regulation of cellular proliferation, death, and the immune response. These help to ultimately insure proper propagation of the virus in the face of a hostile cellular environment and to manipulate the host machinery in the interest of viral replication. Some adenoviral proteins, such as *E1A* and *E4* gene products, promote host cell proliferation by driving an otherwise stationary or slow cell cycle, thus providing an optimal

*Abbreviations:* SR proteins, serine/arginine-rich proteins; ADP, adenovirus death protein; Rb, retinoblastoma protein; TNFα, tumor necrosis factor α; CAPP, ceramide-activated protein phosphatase; MOI, multiplicity of infection; pfu, plaque-forming unit; PARP, poly(ADP)-ribose polymerase.

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environment for viral replication (Leppard, 1997; Nevins, 1995). In the case of E1A, this is accomplished by targeting key cellular proteins that control cell cycle such as the retinoblastoma protein (Rb) (Dyson et al., 1989; Mal et al., 1996; Whyte et al., 1989). Other adenoviral genes, such as *E1B19K*, *E1B55K*, and most of the *E3* genes, encode for proteins whose function is to help the virus evade the innate immune response and to prevent early apoptosis by inhibiting the tumor suppressor p53 and other apoptotic pathways, thus delaying the elimination of infected cells long enough until sufficient viral progeny is produced (Gooding, 1992; Han et al., 1998; Perez and White, 1998; Wold et al., 1999).

Among the emerging regulators of cell proliferation and apoptosis is ceramide, a putative coordinator of the cellular stress response by virtue of its ability to induce cell cycle arrest by regulating Rb and to induce p53-independent apoptosis (Dbaibo et al., 1995; Jayadev et al., 1995; Obeid et al., 1993). Thus, ceramide shares functional properties with some targets of adenoviral genes, namely, Rb and p53. However, the targeting of ceramide pathways by adenovirus has not been examined.

The cellular levels of ceramide are regulated by the balance of the rates of its de novo synthesis that begins with the condensation of serine and palmitoyl-CoA, its hydrolysis to sphingosine and fatty acid, its incorporation into more complex sphingolipids, and its generation from the hydrolysis of these compounds (Hannun and Obeid, 2002). Inducers of ceramide accumulation include tumor necrosis factor α (TNFα), Fas ligation, serum deprivation, heat shock, and ionizing irradiation (Chang et al., 1995; Haimovitz-Friedman et al., 1994; Jayadev et al., 1995; Kim et al., 1991; Tepper et al., 1995). After stimulation with one of the inducers, ceramide levels begin to increase over a period of minutes or hours, and, depending on the cell type and inducer, cells undergo differentiation, cell cycle arrest, senescence, or apoptosis. Downstream effector targets of ceramide include ceramide-activated phosphatases (CAPP) and, less directly, several ceramide-activated kinases (Dobrowsky and Hannun, 1992; Liu et al., 1994; Zhang et al., 1997). The first CAPP identified is a member of the 2A family of serine/threonine protein phosphatases and was shown to be an effector molecule for ceramide-induced biology (Wolff et al., 1994). PP2A was found in yeast *Saccharomyces cerevisiae* to mediate ceramide-induced growth arrest (Fishbein et al., 1993). Another more recently identified CAPP is PP1 that was shown to be potently stimulated by naturally occurring ceramides and to mediate ceramide-induced caspase activation (Chalfant et al., 1999; Kishikawa et al., 1999). Among the PP1 substrates that were identified, serine/arginine-rich (SR) proteins emerged as the most relevant to the apoptotic functions of ceramide. These proteins are regulated by phosphorylation and play an important role in alternative splicing of several apoptosis regulatory genes in response to external death stimuli (Chalfant et al., 2001). In this study, we examine the effects of adenoviral infection on ceramide pathways and the role it plays during infection.

## Results

### *Adenovirus infection induces ceramide accumulation*

The effects of adenovirus infection on the ceramide response were examined. Infection of MCF7 cells with increasing multiplicity of infection (MOI) of adenovirus rec700 (wild type) was done (Fig. 1A). At 48 h, cells were harvested, and ceramide levels were measured and compared to time-matched controls. It was found that ceramide levels increased with increasing MOI, reaching a plateau of about 4-fold of baseline levels at an MOI of 80 pfu/cell. The time course of ceramide increase following infection was then examined (Fig. 1B). Infection with rec700 at 160 pfu/cell resulted in a gradual increase in ceramide starting at 24 h and increasing steadily with time to reach 4-fold of baseline levels by 48 h. When lower MOIs were examined, we found that, at an MOI of 20 pfu/cell, ceramide levels started to increase at 48

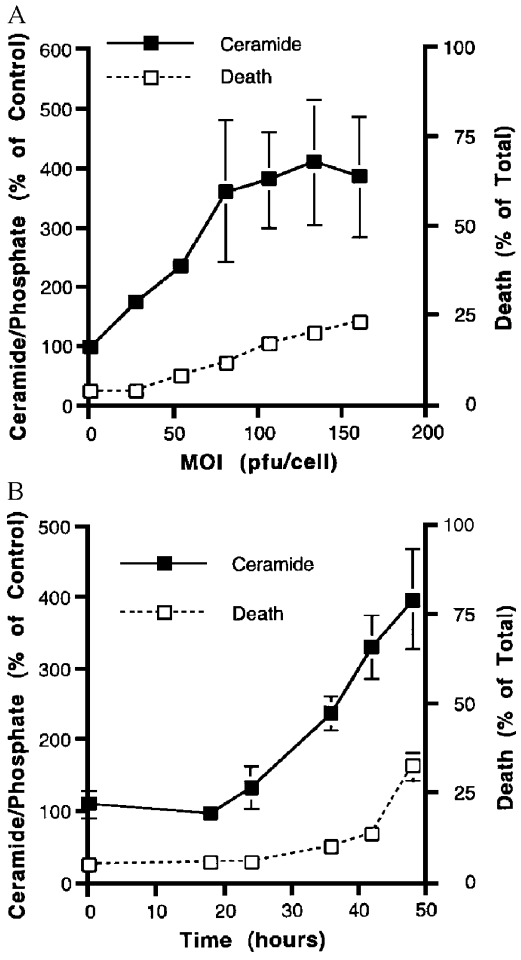


Fig. 1. Effect of adenovirus infection on ceramide levels and cell lysis. (A) Dose-dependent increase in ceramide and cell lysis. MCF7 cells were infected with rec700 adenovirus at the indicated MOIs. Cells were harvested at 48 h post-infection, and ceramide was measured as described in Materials and methods. Cell lysis was determined using the trypan blue exclusion assay. (B) Time-dependent increase in ceramide and cell lysis. MCF7 cells were infected with adenovirus rec700 as in panel A at an MOI of 160 pfu/cell and harvested at the indicated time points. Ceramide levels and cell lysis were measured as in panel A. Data are representative of at least three independent experiments.

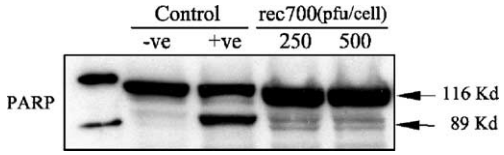


Fig. 2. Lack of PARP cleavage during adenovirus infection. MCF7 cells were either mock-infected (−ve Control) or infected for 48 h with rec700 adenovirus at an MOI of 250 pfu/cell or 500 pfu/cell. Total protein lysates (100 μg) were subjected to 10% SDS-PAGE analysis and immunoblotted for PARP protein. Positive control for PARP cleavage was used consisting of lysates from MCF7 cells treated with TNFα for 24 h. The cleaved "apoptotic" fragment is indicated at 89 kDa.

h and reached 3-fold of baseline levels by 96 h (data not shown). Similar results were obtained when A549 lung adenocarcinoma cells were used (data not shown). These findings suggested that adenoviral infection was associated with a significant accumulation of ceramide.

### *Relationship of accumulated ceramide with adenovirus-induced cell death*

Due to the demonstrated association between ceramide accumulation and various forms of cell death including apoptosis, we investigated the relationship of ceramide and cell death during adenoviral infection. In human cells, adenovirus induces a lytic infection where mature virions are released from the infected cell at the end of the viral replication cycle. In order to determine whether cell lysis occurred before, with, or after ceramide accumulation, MCF7 cells were infected with rec700 adenovirus at several doses and were analyzed for cell death using the trypan blue exclusion assay at 48 h post-infection and compared to mock-infected controls. At the lower doses, adenovirus caused no cell death at 48 h, although significant ceramide accumulation had occurred with these doses (Fig. 1A). The time course of cell death following adenoviral infection was examined next. Infection of MCF7 cells with rec700 at 160 pfu/cell was performed and cell death determined at the indicated time points. There was no significant death in MCF7 cells by 24 h post-infection as compared to controls. By 36 h post-infection, about 10% of the cells were dead, and cell death continued to rise in a time-dependent manner, reaching 30% by 48 h post-infection (Fig. 1B). These findings indicated that ceramide accumulation occurred prior to cell lysis during adenoviral infection in a closely related manner.

In several systems, ceramide has been shown to induce apoptosis. Therefore, we examined whether, during adenoviral infection, ceramide played a role in the regulation of viral-induced apoptosis. Adenovirus encodes for several anti-apoptotic proteins such as E1B 19K and E1B 55K that function to prevent or delay apoptosis in order for the viral replication cycle to be completed. Therefore, apoptosis following infection of MCF7 cells with rec700 adenovirus was examined by assaying for poly(ADP)-ribose polymerase (PARP) cleavage from its native 116 kDa to an "apoptotic" fragment of 89 kDa by the specific action of caspases. Infection with rec700 at an MOI of 250 or 500 pfu/cell resulted in cell death as measured by trypan blue uptake of 46% and 64%, respectively. This occurred in the absence of significant PARP cleavage, suggesting that non-apoptotic cell death was induced during adenoviral infection (Fig. 2). In comparison, treatment of MCF7 cells with 3 nM TNFα for 24 h resulted in cell death of 65% and significant PARP cleavage. Therefore, ceramide accumulation during adenoviral infection was accompanied by non-apoptotic cell death.

### *Ceramide accumulates in the absence of cell lysis*

Since ceramide accumulation was accompanied by cell lysis that lacked biochemical features of apoptosis, it was important to verify that ceramide accumulation was not caused by nonspecific activation of biochemical pathways that generate ceramide during cell lysis. Thus, a mutant adenovirus dl7001 that lacks the E3 gene cassette was utilized. This virus lacks the E3 11.6 K adenovirus death protein (ADP) that is essential for cell lysis and viral release. Cell death in MCF7 cells infected with 500 pfu/cell of dl7001 was determined using the trypan blue exclusion assay. Infection induced only 7% death in the MCF7 cells as compared to 5% of mock-infected control cells at 36 h post-infection with no significant rise at later time points (Fig. 3). However, when ceramide levels were measured in the MCF7 cells infected with the mutant virus, they were found to have significantly increased levels, comparable to those achieved with wild type virus infection (Fig. 3). Similar results were obtained when A549 cells or when lower MOIs and longer time points were used (data not shown). These findings lead to two conclusions relating to ceramide accumulation during adenoviral infection: 1) ceramide did not accumulate due to nonspecific activation of relevant biochemical pathways during cell lysis, and 2) ceramide accumulation did not necessarily result in cell death.

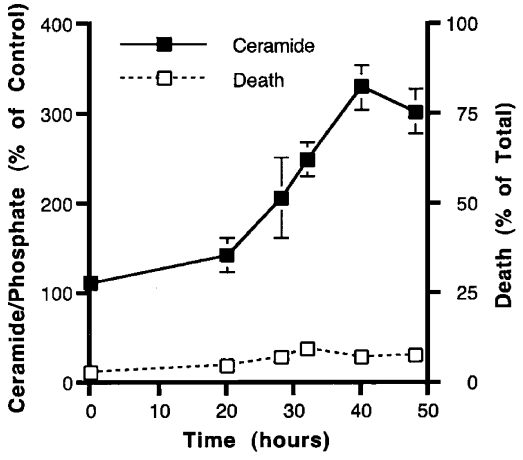


Fig. 3. Ceramide accumulates in the absence of cell lysis after infection with an E3 mutant adenovirus. MCF7 cells were infected with adenovirus dl7001 that lacks all the E3 genes at an MOI of 500 pfu/cell. Ceramide levels and cell lysis were determined as in Fig. 1B.

*Adenoviral infection induces de novo synthesis of ceramide*

Several biochemical pathways are involved in regulating cellular ceramide levels. Activation of sphingomyelinases results in the hydrolysis of membrane sphingomyelin and generation of ceramide. Inhibition of enzymes that metabolize ceramide such as ceramidases and glucosylceramide synthase also results in the accumulation of cellular ceramide. Alternatively, ceramide can be generated by de novo synthesis from the condensation of serine and palmitate. Several stimuli were demonstrated to activate one or more of these pathways in many cell types. In MCF7 cells, TNFα was shown to activate magnesium-dependent neutral sphingomyelinase to generate a modest increase in ceramide at early time points and to activate de novo synthesis to generate more elevated and sustained levels of ceramide at later time points (Dbaibo et al., 2001). Adenoviral infection of MCF7 cells induced late accumulation of ceramide. This raised the possibility that the de novo synthesis pathway was involved. In order to examine this possibility, we used the ceramide synthase inhibitor Fumonisin B1. When MCF7 cells were treated with Fumonisin B1 [100 μM] at the time of infection with the wild type adenovirus rec700, the increase in ceramide between 24 and 48 h post-infection was completely inhibited as compared to time-matched controls (Fig. 4A). Similar results were obtained when the serine–palmitoyl transferase inhibitor Myriocin was used (Fig. 4A). This suggested that ceramide accumulation in response to adenoviral infection was mainly derived from the de novo synthesis pathway.

To directly assess the role of the de novo ceramide biosynthesis in cells in response to adenoviral infection, MCF7 cells were labeled with [$^3$H]-palmitate and then infected with wild type adenovirus with and without co-treatment with Fumonisin B1 or Myriocin. An increase in the incorporation of palmitate into ceramide upon wild type adenoviral infection was found, starting as early as 24 h and sustained at 48 h post-infection, as compared to controls. As expected, treatment with Fumonisin B1 or Myriocin completely abrogated the incorporation of palmitate into the complex sphingolipids including ceramide (Figs. 4B and C). Comparable results were obtained when we radiolabeled A549 cells (data not shown). These studies supported a major role for de novo synthesis of ceramide as a source of accumulated ceramide during adenoviral infection.

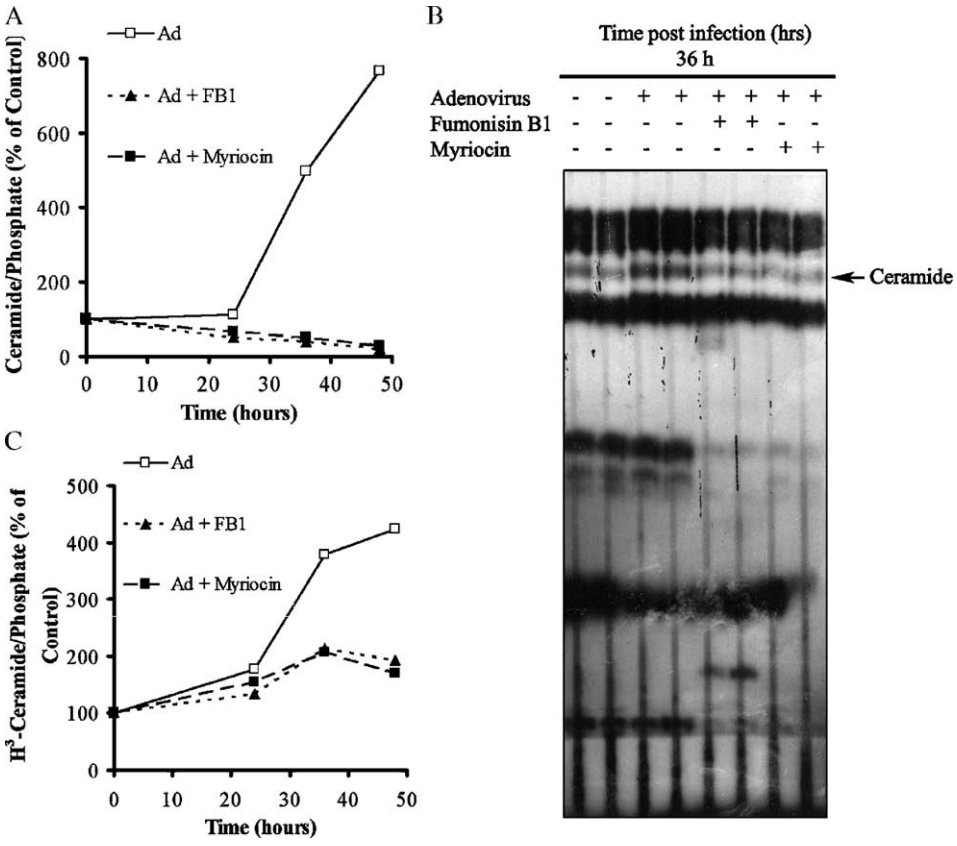


Fig. 4. Ceramide accumulates by de novo synthesis during adenoviral infection. (A) Inhibitors of de novo ceramide synthesis block ceramide accumulation during adenoviral infection. MCF7 cells were treated with Fumonisin B1 100 μM (FB1) or Myriocin 50 nM at the time of infection with rec700 adenovirus (Ad). Cells were harvested at 24, 36, and 48 h post-infection, and ceramide was measured. (B and C) Evidence of de novo synthesis of ceramide by [$^3$H]-palmitate labeling during adenoviral infection. MCF7 cells were pulse labeled with 1 μCi/ml [$^3$H]-palmitate at the time of infection with rec700 adenovirus in the presence or absence of Fumonisin B1 or Myriocin. Cells were harvested at the indicated time points. Lipids were extracted, and the newly synthesized ceramide band was separated on TLC following base hydrolysis as described in Materials and methods. The TLC from the 36-h time point is shown in panel B. Quantitation of newly synthesized ceramide is shown in panel C.

*Inhibition of de novo synthesis of ceramide delays lysis of infected cells*

Ceramide has been implicated in the regulation of apoptosis as well as necrosis. Whereas lysis of adenovirus-infected cells lacks biochemical or morphological features of apoptosis, there is evidence that it is a regulated process that is dependent, at least in part, on viral ADP (Tollefson et al., 1996). In order to determine whether ceramide plays a role in lysis of infected cells, we utilized the inhibitors of de novo ceramide synthesis Fumonisin B1 and Myriocin. Infection of MCF7 cells was performed in the presence of either of the ceramide synthesis inhibitors, and cells were evaluated at 24, 36, and 48 h by phase contrast microscopy (Fig. 5A) and by uptake of trypan blue (Fig. 5B). It was found that both inhibitors were capable of significantly delaying cell lysis as reflected by cell rounding, detachment, and loss of membrane integrity. These studies suggested that the accumulation of ceramide was a necessary step in cell lysis during infection.

*Adenovirus-induced dephosphorylation of SR proteins is regulated by de novo synthesized ceramide*

During adenoviral infection, lysis of infected cells occurs after completion of viral replication and is dependent on ADP for efficient release of virions. During the late stages of viral replication cycle, alternative splicing of the L1 gene results in the shift from the 52,55 K mRNA produced early in the infectious cycle to the IIIa mRNA that is predominant in the late stages of the cycle (Kanopka et al., 1996). This process is regulated by cellular spliceosomes that process pre-mRNA into mature mRNA. SR proteins are important components of spliceosomes. A subset of SR proteins is specifically targeted by adenovirus via its E4orf4 splicing enhancer protein, which

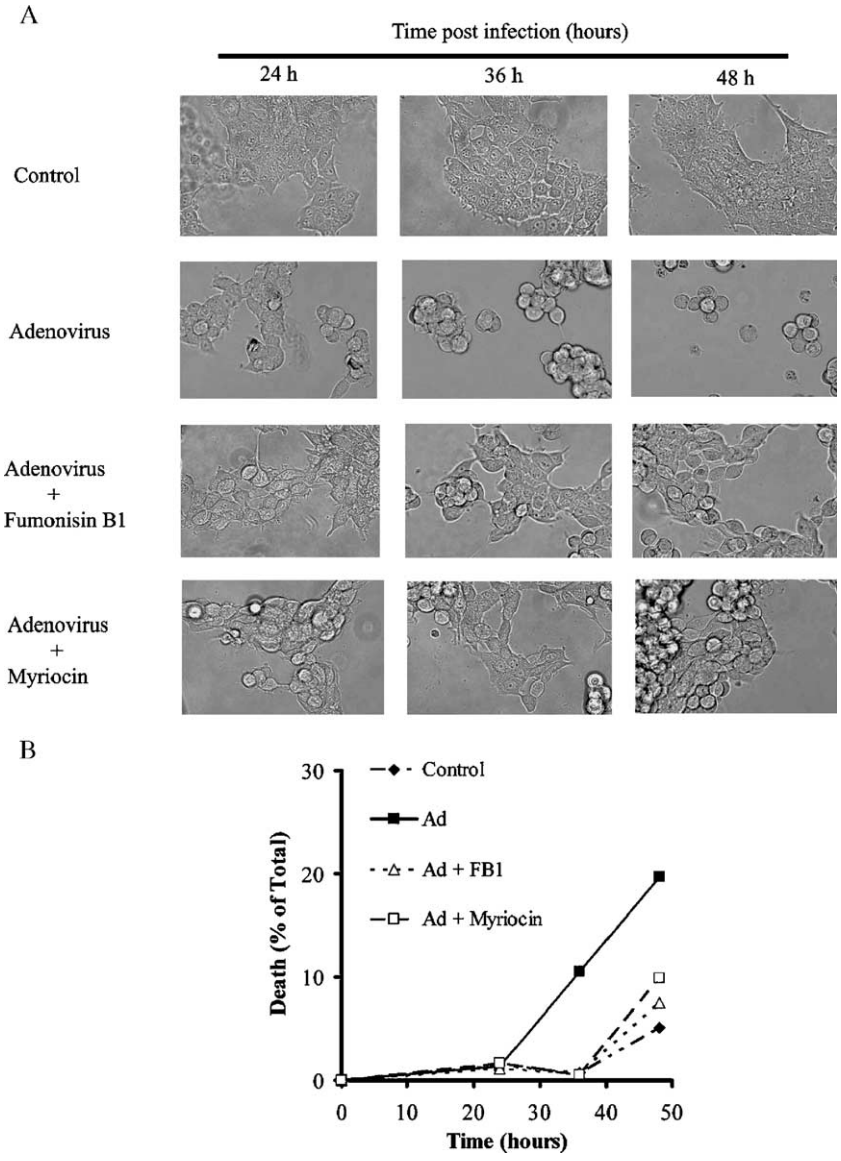


Fig. 5. Inhibition of ceramide synthesis delays cell lysis during adenoviral infection. MCF7 cells were infected with rec700 adenovirus (Ad) at an MOI of 160 pfu/cell as in Fig. 1 in the presence or absence of Fumonisin B1 100 μM (FB1) or Myriocin 50 nM at the time of infection and evaluated at the indicated time points. (A) Phase contrast microscopy. (B) Determination of cell lysis by trypan blue exclusion.

activates cellular protein phosphatase 2A (PP2A) to induce dephosphorylation of SR proteins (Estmer Nilsson et al., 2001; Kanopka et al., 1998; Marcellus et al., 2000). Ceramide generated by the de novo synthesis pathway was recently implicated in the regulation of SR proteins by virtue of its activation of protein phosphatase 1 (PP1), which also targets SR proteins as substrates (Chalfant et al., 2001, 2002). Therefore, we examined whether ceramide generated by de novo synthesis during adenoviral infection was involved in SR protein regulation. Using an antibody that recognizes a phosphoepitope common to all SR proteins, it was found that a subset of SR proteins that includes SR p75, SR p55, SR p40, SR p30, and SR p20 was dephosphorylated in adenoviral-infected MCF7 or A549 cells, a response detected at 48 and 72 h post-infection coinciding with the significant rise in ceramide accumulation during infection (Fig. 6). When cells were treated with Myriocin at the time of infection, dephosphorylation was significantly inhibited (Fig. 6A). Similar results were obtained when Fumonisin B1 was used in A549 (Fig. 6B) or MCF7 (Fig. 6C) cells. These data indicated that the induction of de novo ceramide synthesis during adenoviral infection plays a significant role in the dephosphorylation of SR proteins.

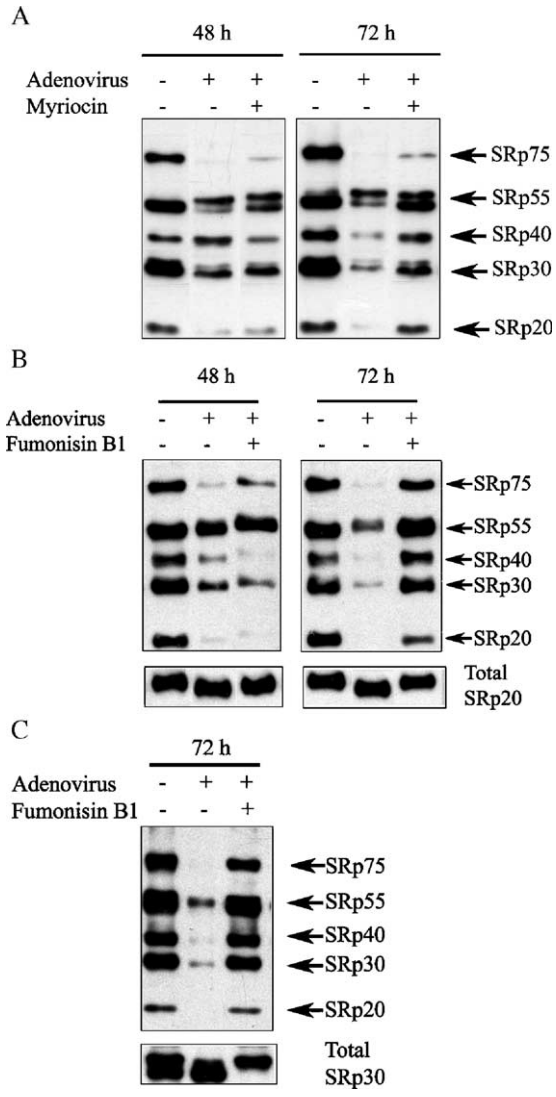


Fig. 6. Adenovirus-induced ceramide regulates the phosphorylation state of SR proteins. (A) Myriocin inhibits adenoviral-induced dephosphorylation of SR proteins. A549 cells were infected with rec700 adenovirus (Ad) in the presence or absence of Myriocin 50 nM, and total protein lysates were prepared at the indicated time points and subjected to Western blotting using the SR phospho-specific antibody mAb104. Phosphorylated SR proteins are indicated with closed arrows. (B and C) Fumonisin B1 inhibits adenoviral-induced dephosphorylation of SR proteins in A549 (B) and MCF7 (C) cells. A549 (B) or MCF7 (C) cells were infected with rec700 adenovirus in the presence or absence of Fumonisin B1 100 μM, and total protein lysates were prepared at the indicated time points. Immunoblotting for phosphorylated SR proteins (mAb104), for total SRp30 (ASF/SF2), or for total SRp20 was performed. Total SR proteins are indicated.

## Discussion

In this study, we demonstrate that ceramide levels increase in response to adenoviral infection in at least two different cell types. This increase starts several hours after the expression of early viral genes such as E1A (data not shown) and precedes any evidence of cell lysis. Moreover, the increase in ceramide occurs in the absence of cell lysis as shown in Fig. 3 following infection with dl7001 that lacks all E3 genes including ADP. These findings indicate that ceramide accumulation during adenoviral infection is not a result of cell lysis with nonspecific activation of ceramide-generating pathways nor is it due to the expression of ADP. In addition, the accumulated ceramide is generated mostly by de novo synthesis. The fact that an infected and dying cell is spending valuable energy to actively synthesize ceramide suggests an important role that ceramide plays during adenoviral infection.

In other systems, such as the treatment of MCF7 cells with TNFα or the treatment of A549 cells with chemotherapeutic agents, ceramide elevation was followed shortly by apoptosis with typical morphological and biochemical features (Dbaibo et al., 1997; Ogretmen et al., 2001). However, in the current study, it was found that infection with wild type adenovirus resulted in ceramide elevation that was followed by non-apoptotic cell lysis. The lack of apoptosis was most likely due to the expression of the *E1B19K* gene product. E1B19K is a viral homolog of the cellular anti-apoptotic protein Bcl-2 and shares its ability to inhibit apoptosis (Chiou et al., 1994). This is accomplished by binding to several of the pro-apoptotic members of the Bcl-2 family including Bax and Bak and inhibiting the mitochondrial release of cytochrome C (Han et al., 1998; Perez and White, 1998). Bcl-2 was shown, in several systems, to function “downstream” of ceramide. For example, MCF7 cells that overexpress Bcl-2 were not only resistant to apoptosis induced by TNFα but were also resistant to ceramide-induced apoptosis (Dbaibo et al., 1997; El-Assaad et al., 1998). In response to TNFα, these cells accumulated significant levels of ceramide without undergoing apoptosis. Thus, it is reasonable to speculate that the expression of E1B19K during adenoviral infection may act downstream of ceramide to block ceramide-induced apoptosis.

Two lines of evidence from this study strongly suggest that the bulk of the accumulated ceramide was produced from the de novo biosynthetic pathway. First, the activities of ceramide

synthase and serine–palmitoyl transferase were required for ceramide accumulation. Inhibition of either enzyme by Fumonisin B1 or Myriocin, respectively, resulted in significant attenuation of ceramide increase (Desai et al., 2002; Perry, 2000). Second, adenoviral infection caused active de novo ceramide production as demonstrated by palmitate labeling studies. The fact that either Fumonisin B1 or Myriocin was capable to significantly delay cell rounding, detachment, and lysis as compared to wild type infected cells suggests that ceramide generated by the de novo pathway is required for completion of the infectious cycle and lysis of the infected cells. A limitation of these experiments though is that treatment with Fumonisin B1 or Myriocin for prolonged periods, in addition to preventing ceramide synthesis, results in the depletion of sphingolipids derived from ceramide. Thus, depletion of more complex sphingolipids might contribute to the delayed lysis observed after Fumonisin B1 or Myriocin treatment.

The lack of apoptosis after ceramide accumulation combined with the delay in cell lysis after adenoviral infection when ceramide synthesis was inhibited raised questions regarding the role of ceramide accumulation during adenoviral infection. Was ceramide increased simply as a "stress response" to viral infection? In this regard, was ceramide one of the cellular weapons to counteract the effects of adenoviral genes to promote cell proliferation and prevent apoptosis? Alternatively, was ceramide elevation driven by adenovirus in order to accomplish specific effects that are necessary for completion of the adenoviral lytic cycle? Previously, de novo synthesized ceramide was shown to regulate SR protein function, and this raised the possibility that ceramide may regulate alternative splicing during adenoviral infection.

SR proteins are serine/arginine-rich proteins that constitute a family of pre-mRNA splicing factors (Manley and Tacke, 1996). They are involved in multiple steps of the constitutive splicing reaction by insuring the correct assembly of a functional spliceosome and are important regulators of alternative splicing where they can function either positively or negatively to enhance or repress the utilization of specific splice sites (Furuyama and Bruzik, 2002; Hastings and Krainer, 2001; Lynch and Maniatis, 1995; Smith and Valcarcel, 2000; Tacke and Manley, 1999; Tian and Maniatis, 1992). SR proteins exist in the cell as phosphoproteins where reversible phosphorylation significantly regulates their function (Colwill et al., 1996; Gui et al., 1994; Mermoud et al., 1994). De novo ceramide accumulation in response to Fas activation and heat shock was shown to induce dephosphorylation of SR proteins by activation of protein phosphatase 1 (Chalfant et al., 2001). In the context of Fas treatment, this resulted in alternative splicing of two key apoptotic genes, caspase 9 and Bcl-x. In both cases, ceramide treatment favored the production of the alternatively spliced pro-apoptotic transcripts caspase-$9_L$ and Bcl-$x_s$ (Chalfant et al., 2002). Similarly, alternative splicing by SR proteins was essential during lytic adenoviral infection where SR proteins were involved in the alternative splicing of the adenoviral late L1 gene. Transcription of the gene produces a precursor mRNA that can be alternatively spliced to produce either the 52/55K or the IIIa mRNAs. This is temporally regulated in such a way that the efficient use of the IIIa splice site is confined to the late phase of the cycle (Estmer Nilsson et al., 2001; Molin and Aküsjarvi, 2000). Indeed, the E4orf4 adenoviral gene product was shown to associate with PP2A, forming a complex that was able to induce dephosphorylation of SR proteins and hence regulate the temporal shift in adenovirus alternative RNA splicing (Estmer Nilsson et al., 2001; Kanopka et al., 1998). Thus, the findings from the current study that ceramide was necessary for dephosphorylation of SR proteins during adenoviral infection suggest that, in addition to the E4orf4/PP2A pathway, adenovirus may utilize the ceramide/PP1 pathway to regulate SR protein function.

Our findings begin to define a role for ceramide during adenoviral infection and raise the possibility that adenovirus might hijack the ceramide pathway in order to promote cellular or viral alternative splicing in the late phase of the viral cycle.

## Materials and methods

### *Materials*

MCF7 M1 breast carcinoma epithelial cells were previously obtained from Vishva Dixit, University of Michigan, Ann Arbor, A549 lung carcinoma cells were a kind gift from Besim Ogretmen Medical University of South Carolina, Charleston. Adenovirus rec700 (recombinant of adenovirus types 2 and 5) and $E3^-$ dl7001 (deleted in all of the E3 transcription unit) were described previously (Gooding et al., 1991). Myriocin was from Sigma. Fumonisin B1 was from Alexis. ENHANCE was from Perkin-Elmer Life Sciences. [$\gamma$-$^{32}$P]-ATP and [9,10-$^{3}$H(N)]-palmitate were from Amersham Biosciences. mAb104 (phospho-SR protein antibody) hybridoma cells were purchased from ATCC. ASF/SF2 (SRp30) and SRp20 antibodies were from Santa Cruz.

### *Cell culture*

MCF7 M1, breast carcinoma epithelial cells, and A549 lung carcinoma epithelial cells were maintained in RPMI 1640 and DMEM (GIBCO), respectively, containing 10% fetal bovine serum (GIBCO) at 37 °C in 5% $CO_2$. For experimental studies, cells were prepared by seeding $3 \times 10^5$ cells in 6 ml of medium containing 10% fetal bovine serum and resting them overnight before infection. Infection studies were performed using serum-free medium for 75 min, and then volume was completed to 10% fetal bovine serum.

### *Growth studies*

Cells were harvested at the indicated time points from cultures, and aliquots were diluted with equal volumes of trypan blue solution. Both trypan blue negative and positive

cells were counted, and the percentage of dead cells was determined.

*Ceramide measurements*

Lipids were collected according to the method of Bligh and Dyer. Ceramide was measured with a modified diacylglycerol kinase assay using external ceramide standards as described. Briefly, 80% of the lipid sample was dried under $N_2$. The dried lipid was solubilized in 20 μl of an octyl-β-D-glucoside/dioleoyl phosphatidylglycerol micellar solution (7.5% octyl-β-D-glucoside, 25 mM dioleoyl phosphatidylglycerol) by several cycles of sonication in a bath sonicator followed by resting at room temperature for 15–20 min. The reaction buffer was prepared as a 2× solution, containing 100 mM imidazole HCL, pH 6.6, 100 mM LiCl, 25 mM $MgCl_2$, and 2 mM EGTA. To the lipid micelles, 50 μl of 2× reaction buffer was added, 0.2 μl of 1 M dithiothreitol, 5 μg of diglycerol kinase membranes, and dilution buffer (10 mM imidazole, pH 6.6, 1 mM diethylenetriaminepentaacetic acid, pH 7) to a final volume of 90 μl. The reaction was started by adding 10 μl 2.5 mM [γ-$^{32}$P]ATP solution (specific activity of 75,000–200,000 cpm/nmol). The reaction was allowed to proceed at 25 °C for 30 min. Bligh and Dyer (1959) lipid extraction was done, and a 1.5 ml aliquot of the organic phase was dried under $N_2$. Lipids were then resuspended in a volume of 50 μl methanol:chloroform (1:9, v/v), and 25 μl was spotted on a 20 cm silica gel thin layer chromatography plate. Plates were developed with chloroform:acetone:methanol:acetic acid:$H_2O$ (50:20:15:10:5), air dried, and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide–phosphate, the phosphorylated products of diacylglycerol and ceramide, respectively, were identified by comparison to known standards. Spots were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted on a scintillation counter. Linear curves of phosphorylation were produced over a concentration range of 0–960 pM of external standards (dioleoyl glycerol and CIII ceramide; Sigma). Ceramide levels were always normalized to lipid phosphate, which was measured according to the method of Rouser et al. (1970). It is important to note that, under these conditions, there was total conversion of ceramide and diacylglycerol to their phosphorylated products, and there was no change in the specific activity of the diacylglycerol kinase enzyme.

For assessing de novo ceramide synthesis, labeling with palmitate was utilized. Cells were seeded at $3 \times 10^5$ cells/well in 6-well culture plates in RPMI with 10% FBS (3.5 ml of media/well). Cells were allowed to grow for 24 h and then were infected in serum-free RPMI medium for 75 min in the presence of 5 μCi/well of [9,10-$^3$H(N)]-palmitate after which the volume was completed to provide a final concentration of 10% FBS. At 18, 24, and 48 h, cells were harvested, washed once in ice-cold PBS, and lipids extracted according to Bligh and Dyer. One third of lipid extract was used for lipid phosphate determination. The other two thirds were base-hydrolyzed and the resulting lipids resolved on TLC (solvent system of chloroform:methanol:2 N $NH_4OH$; 40:10:1). Plates were dried, sprayed with ENHANCE, and exposed to film and $^3$H-lipids were identified. The lipid co-migrating with a ceramide standard was scraped from the TLC plate and quantified by liquid scintillation spectroscopy. Results are expressed as cpm of ceramide/nmol lipid-Pi.

*Western blotting*

Cells were harvested by centrifugation at 1500 rpm for 10 min and washed in ice-cold PBS. Proteins were extracted by direct lysis with sample buffer and were separated on a 10% and 12% SDS-PAGE gels for PARP and SR proteins, respectively and then transferred to a nitrocellulose membrane. Blots were blocked with 5% milk in PBS containing 0.3% Tween 20 and then incubated with the corresponding primary antibody, 1:1000 for PARP, 1:25 for mAb104, and 1:100 for ASF/SF2 and SRp20 antibodies. Bands were developed using the enhanced chemiluminescence detection system (ECL, Amersham Biosciences).

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