why metaphase arrest is seen only in one specific type of cell cycle—meiosis II in vertebrates—when both kinases are active.

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14. Sense and antisense oligodeoxynucleotides were synthesized to three regions of the *Xenopus Cdk2* gene (*8*) not conserved in *Xenopus Cdc2: Cdk2* 227 to 241, ATTCATACGGAAAAC; *Cdk2* 302 to 328, AACATTTCTGGAATTTCATTGCCCCTA; and *Cdk2* 749 to 769, ATCCGACAGGACTTTAGCAAA. Two sets were synthesized, one set as unmodified oligodeoxynucleotides with an Applied Biosystems 394 DNA synthesizer, and the second set with amidate-modified internucleoside linkages (Integrated DNA Technologies, Coralville, IA). Oligodeoxynucleotides were purified by high-performance liquid chromatography before use. Mixtures (50 nl) of the unmodified sense and antisense oligodeoxynucleotides (1 mg/ml each) or the amidate-modified oligodeoxynucleotides (0.3 mg/ml each) were injected into oocytes. Mock-injected oocytes served as controls in some experiments. Oocytes were then incubated in modified OR2 [83 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM KCl, 20 mM Hepes (pH 7.8)] for up to 4 hours at room temperature. Maturation was then induced in OR2 supplemented with 10 mM sodium bicarbonate (pH 7.8), bovine serum albumin (0.1 mg/ml), 1 mM sodium pyruvate, and 10 μM progesterone.
15. At the times indicated in the figures, four oocytes were removed, homogenized in modified EB [15 μl per oocyte (*9*)], and centrifuged for 5 min in a microcentrifuge. The supernatants were stored at −70°C until analyzed. The Cdc2 activity was measured either by precipitation of activity with p13-Sepharose from the total sample [more than 90% attributable to Cdc2 (*9*)], by specific immunoprecipitation with affinity-purified antibody to the COOH-terminus of Cdc2, or as total H1 kinase activity as described (*9*). Cdk2 protein kinase activity was measured in immune-complex kinase assays exactly as described (*9*). Activity is expressed as picomoles of $^{32}P_i$ incorporated per 15 oocytes in 20 min for immunocomplex assays, and picomoles of $^{32}P_i$ incorporated per 0.3 oocytes in 20 min for total activity precipitated with p13-Sepharose. Protein immunoblotting was done with an ECL detection kit (Amersham) except for cyclin B2, which was detected with an alkaline phosphatase color reagent (Bio-Rad). Antibodies to Cdk2 and cyclin B2 were prepared as described (*9*, *23*). Antibody to Cdc2 was raised in rabbits to a 15-residue synthetic peptide from the COOH-terminus of the deduced sequence coupled to keyhole limpet hemocyanin (KLH), and antibodies to c-Mos^{xe} were to a 18-residue synthetic peptide encoding residues 6 to 24 of the deduced sequence also coupled to KLH (*23*). The antibodies were affinity-purified on a column of immunogen peptide coupled to thiopropyl–Sepharose 6B (Pharmacia).
16. Recombinant monomeric Cdk2 was purified from baculovirus-infected Sf9 cells (5 g) expressing full-length *Xenopus* Cdk2. The cells were sonicated in 20 ml of buffer [20 mM tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP-40] containing 0.5 mM phenylmethylsulfonyl fluoride and pepstatin A, leupeptin, chymostatin, and aprotinin (each at 10 μg/ml) and then centrifuged at 15,000*g* for 20 min. The supernatant was then diluted with two volumes of buffer A [20 mM tris (pH 7.0), 2 mM EDTA, 1 mM dithiothreitol] containing 1 mM CHAPS and applied to a 20-ml Matrex Green A gel column (Amicon). The column was washed with the same buffer and eluted with a 500-ml gradient of 0 to 1 M NaCl in buffer A without CHAPS. Fractions were collected and assayed for Cdk2 by immunoblotting. The pooled Cdk2 fraction was made 1 M in ammonium sulfate and applied to a 5-ml phenyl-Sepharose column. The column was washed with buffer A containing 1 M ammonium sulfate, then a 100-ml gradient of 1 to 0 M ammonium sulfate was applied. The column was then washed with 30 ml of buffer A and Cdk2 was eluted with 40% (v/v) ethylene glycol in buffer A. The Cdk2 fraction was diluted with three volumes of buffer A containing 0.01% Brij-35 and passed over a fast protein liquid chromatography (FPLC) Mono Q column (Pharmacia). Cdk2 eluted in the unbound fraction and was further concentrated on a Pharmacia SMART System Mono S column. At this stage the monomeric Cdk2 was visible by silver staining as a major band, but had no detectable kinase activity (*9*).
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19. The catalytically active, multi-component complex of Cdk2 was purified from unfertilized eggs by a protocol similar to that for purification of the monomer (*16*) except that the Brij-35 concentration was increased to 0.05% for chromatography on the FPLC Mono Q and SMART System Mono S columns. The final preparation was highly purified as judged by silver staining and contained no detectable Cdc2. Unlike monomeric Cdk2, the active complex eluted in the unbound fraction on the SMART system Mono S column. The Cdk2 H1 kinase complex purified to this stage and CHX (100 μg/ml) were injected (50 nl) into progesterone-stimulated oocytes just as white spots began to form.
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Programmed Cell Death Induced by Ceramide

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Sphingomyelin hydrolysis and ceramide generation have been implicated in a signal transduction pathway that mediates the effects of tumor necrosis factor–α (TNF-α) and other agents on cell growth and differentiation. In many leukemic cells, TNF-α causes DNA fragmentation, which leads to programmed cell death (apoptosis). C_2-ceramide (0.6 to 5 μM), a synthetic cell-permeable ceramide analog, induced internucleosomal DNA fragmentation, which was inhibited by zinc ion. Other amphiphilic lipids failed to induce apoptosis. The closely related C_2-dihydroceramide was also ineffective, which suggests a critical role for the sphingolipid double bond. The effects of C_2-ceramide on DNA fragmentation were prevented by the protein kinase C activator phorbol 12-myristate 13-acetate, which suggests the existence of two opposing intracellular pathways in the regulation of apoptosis.

A sphingomyelin cycle has been described in HL-60 leukemia cells in which the action of TNF-α and other inducers of differentiation causes sphingomyelin hydrolysis and ceramide generation (*1–3*). Ceramide in turn seems to be a second messenger that mediates the effects of TNF-α on cell growth and differentiation (*4–6*). In many cell types, TNF-α causes loss of viability

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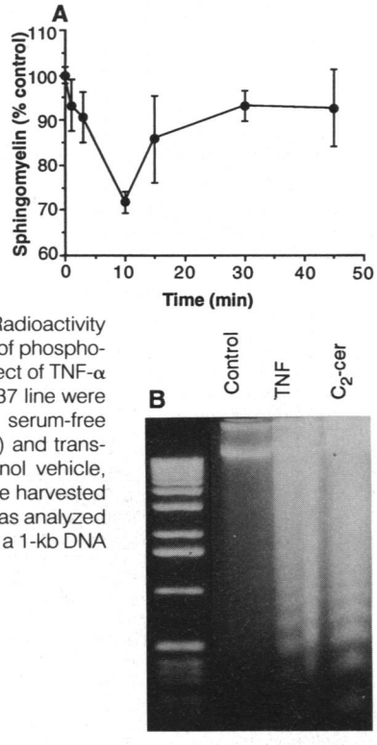

Fig. 1. DNA fragmentation and sphingomyelin hydrolysis in U937 cells. (**A**) Induction of sphingomyelin turnover in response to TNF-α in U937 cells. These cells were grown in RPMI supplemented with fetal calf serum (10%) to 1 × 10^6 cells ml^{-1}. They were then incubated with [^{3}H]choline chloride (0.5 μCi ml^{-1}; specific activity, 80 Ci $mmol^{-1}$; New England Nuclear, Wilmington, Delaware) for 48 to 72 hours. The cells were then washed and treated with TNF-α (30 nM) for the indicated times. Lipids were then extracted, applied to thin-layer chromatography plates, and developed in chloroform-methanol–acetic acid–water (50:30:8:5, v/v). Sphingomyelin spots were visualized by fluorography, scraped, and quantitated by scintillation spectrometry. Radioactivity in the sphingomyelin spots was corrected for the amount of phospholipids. This procedure is described in detail in (*4*). (**B**) Effect of TNF-α and C_2-ceramide on DNA fragmentation. Cells of the U937 line were incubated at a concentration of 2 × 10^5 cells ml^{-1} in serum-free medium (Gibco) supplemented with insulin (5 mg $liter^{-1}$) and transferrin (5 mg $liter^{-1}$). Cells were then treated with ethanol vehicle, TNF-α (10 nM), or C_2-ceramide (C_2-cer, 5 μM). Cells were harvested after 3 hours and DNA was collected (*30*). DNA (10 μg) was analyzed by electrophoresis on a 1.2% agarose gel. The first lane is a 1-kb DNA ladder (Gibco).

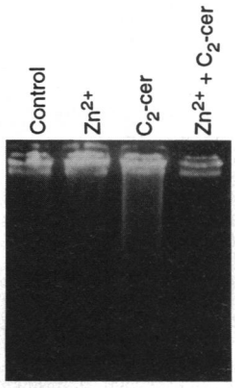

Fig. 3. Inhibition by Zn^{2+} of C_2-ceramide–induced DNA fragmentation. Cells were treated with ethanol vehicle or C_2-ceramide (3 μM) in the presence or absence of 10 μM $ZnSO_4$ for 3 hours. DNA was isolated and analyzed as described (Fig. 1).

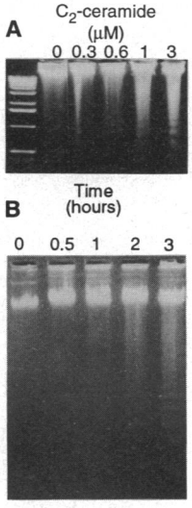

Fig. 2. Effect of ceramide on DNA fragmentation. (**A**) U937 cells were incubated in serum-free medium (Fig. 1B). Cells were treated for 3 hours with ethanol vehicle or with the indicated concentrations of C_2-ceramide. (**B**) U937 cells were treated with C_2-ceramide (3 μM) for the indicated times. DNA was isolated and analyzed as described in Fig. 1.

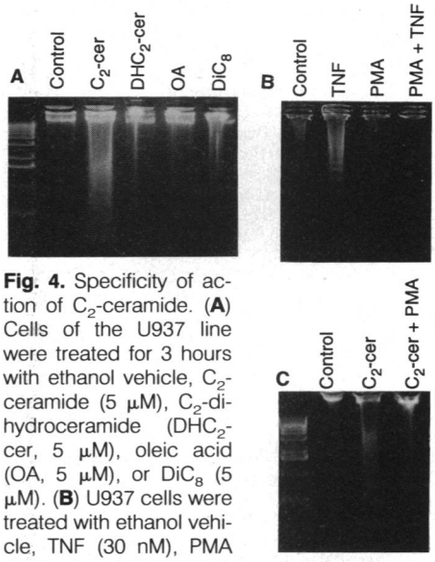

Fig. 4. Specificity of action of C_2-ceramide. (**A**) Cells of the U937 line were treated for 3 hours with ethanol vehicle, C_2-ceramide (5 μM), C_2-dihydroceramide (DHC_2-cer, 5 μM), oleic acid (OA, 5 μM), or DiC_8 (5 μM). (**B**) U937 cells were treated with ethanol vehicle, TNF (30 nM), PMA (100 nM), or PMA (100 nM) and TNF (30 nM) for 3 hours. (**C**) U937 cells were treated with ethanol vehicle, C_2-ceramide (3 μM), or C_2-ceramide (3 μM) and PMA (100 nM) for 3 hours.

that has been attributed to programmed cell death (*7–9*). The mechanism and intracellular mediators of programmed cell death are largely unknown (*10*).

We investigated the hypothesis that ceramide functions as an intracellular mediator of apoptosis induced by TNF-α. We used U937 monoblastic leukemia cells in which TNF-α causes apoptosis (*11, 12*). Exposure of U937 cells to TNF-α (30 nM) resulted in sphingomyelin hydrolysis. Peak effects occurred within 10 to 15 min, and the amounts of sphingomyelin returned to base line within 1 hour (Fig. 1A). The cellular concentrations of ceramide, the product of sphingomyelin hydrolysis, increased by 45% (from 28.2 ± 3 to 40.2 ± 4 pmol $nmol^{-1}$ of phospholipid) at 10 min after the addition of TNF-α (*13*). These results demonstrate that TNF-α activates the sphingomyelin cycle in U937 cells and suggest that ceramide may mediate some of the biologic effects of TNF-α.

To determine whether ceramide itself caused DNA fragmentation, we treated U937 cells for 3 hours with 5 μM C_2-ceramide (a short chain, cell-permeable analog of ceramide) (*5*), ethanol vehicle, or TNF-α (10 nM). Genomic DNA was then obtained from the cells. Treatment of cells with either TNF-α or C_2-ceramide caused DNA fragmentation (Fig. 1B) with a pattern characteristic of internucleosomal fragmentation (*14*), which is considered an early hallmark of programmed cell death (*10, 14*).

Increases in C_2-ceramide concentrations resulted in increased DNA fragmentation (Fig. 2A). The effects of C_2-ceramide (3 μM) were first detected after 2 hours and increased with longer treatment (Fig. 2B). The concentrations of C_2-ceramide that were required to cause DNA fragmentation are similar to the intracellular concentration of ceramide that is generated in response to TNF-α (*4*), and the time course is consistent with a role for C_2-ceramide in mediating the effects of TNF-α.

We obtained further evidence for the similarity of action of TNF-α and C_2-ceramide on DNA fragmentation by investigating the sensitivity of the process to Zn^{2+}. In this cell line, the effects of both C_2-ceramide and of TNF-α were inhibited by 10 μM Zn^{2+} (Fig. 3) (*11*). Taken together, the results in these studies provide evidence for a role of ceramide in mediating the effects of TNF-α.

Because C_2-ceramide is an amphiphilic lipid analog that may have nonspecific activities, it was important to establish its specificity of action. Other amphiphilic lipids such as fatty acids did not cause DNA fragmentation (Fig. 4A). A close structural analog of C_2-ceramide, C_2-dihydroceramide, also failed to induce DNA fragmentation (Fig. 4A). Furthermore, in a quantitative assay of DNA fragmentation (*15*) TNF (20 nM) caused 34 ± 1% DNA fragmentation and C_2-ceramide (3 and 6 μM) caused 27.5 ± 1.5% and 38 ± 2% DNA fragmentation, respectively. Equivalent concentrations of C_2-dihydroceramide were inactive. Because C_2-dihydroceramide differs from C_2-ceramide only in that it lacks the 4,5 trans double bond, these studies demonstrate the specificity of action of C_2-ceramide. The double bond in the sphingoid backbone is probably introduced into sphingolipids after synthesis of dihy-

droceramide from dihydrosphingosine (*16*). Our studies suggest that the introduction of the double bond may be required for ceramide to regulate cell growth and viability.

Because TNF-α may activate other signaling pathways, especially protein kinase C (*17, 18*), we investigated whether activation of protein kinase C resulted in apoptosis in U937 cells. We used dioctanoylglycerol (DiC_8) as a cell-permeable analog of endogenous diacylglycerols as well as phorbol 12-myristate 13-acetate (PMA), a pharmacologic activator of protein kinase C. Neither agent caused DNA fragmentation (Fig. 4, A and B). Although PMA causes internucleosomal DNA fragmentation in the U937 cell line, the effects occur after 12 hours and may be a consequence of cell differentiation (*19*). The faster effects of C_2-ceramide suggest that the sphingomyelin-ceramide pathway may mediate the action of TNF-α on apoptosis. In fact, the addition of PMA prevented the effects of TNF-α and C_2-ceramide on DNA fragmentation (Fig. 4, B and C), which is consistent with reports that PMA inhibits apoptosis (*20, 21*).

These studies identify ceramide as a possible mediator of apoptosis in response to the action of a number of extracellular agents and insults. For example, γ-interferon also causes sphingomyelin hydrolysis and ceramide generation (*4*), and ceramide may mediate its effects on apoptosis. Also, the presence of hypoxia, which induces apoptosis (*22, 23*), results in elevated ceramide concentrations in rat liver in vivo (*24*). Thus, ceramide may also mediate the effects of hypoxia on programmed cell death. Finally, in the human T cell line CEM, which undergoes DNA fragmentation in response to glucocorticoids (*25*), ceramide (3 and 6 μM) induced 57 ± 2% and 76 ± 1% DNA fragmentation, respectively (*15*). Preliminary data also indicate that primary mouse thymocytes undergo DNA fragmentation in response to C_2-ceramide (3 μM). Therefore, the effects of C_2-ceramide are not restricted to U937 cells, and ceramide may mediate apoptosis in response to multiple agents and injuries.

The mechanism of action of ceramide remains poorly understood. Ceramide and sphingosine have been shown to induce phosphorylation of the epidermal growth factor receptor, possibly by activating a protein kinase (*26*). We have recently identified a ceramide-activated protein phosphatase (*27*) that may be involved in transducing the effects of ceramide. The ability of PMA to counteract the effects of ceramide suggests that the effects of the diacylglycerol–protein kinase C pathway counteract those of the proposed ceramide-phosphatase pathway.

Note added in proof: Since submission of this study, Van Veldhoven and colleagues (*28*) have found a significant elevation of ceramide concentrations in human immunodeficiency virus (HIV)–infected CEM cells up to fourfold over concentrations in control uninfected cells. However, phospholipids showed no changes, and diacylglycerol showed only modest increases. Because HIV is known to induce programmed cell death (*29*), the results from the current study suggest that ceramide may mediate the effects of HIV on programmed cell death.

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13. Ceramide concentrations were measured by a modification of the diacylglycerol kinase assay, which quantitates the total cellular mass of ceramide (*5*). The changes in relative and absolute amounts of ceramide in U937 cells closely approximate changes that were observed in HL-60 cells and account for most of the hydrolyzed sphingomyelin (*5*).
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15. Cells of the U937 or CEM lines were incubated with [3H]thymidine (specific activity: 50 Ci $mmole^{-1}$; 20 μCi per 5×10^6 cells) in medium containing 2.5% fetal calf serum for 20 hours. The cells were rinsed three times with serum-free media and then resuspended in serum-free media that contained insulin-transferrin (5 mg $liter^{-1}$) at 0.5×10^6 cells per milliliter. Aliquots (200 μl) were incubated in 24-well plates, and 200 μl of media containing ethanol vehicle or the indicated concentration of inducers was added. After 6 hours the cells were harvested and percent DNA fragmentation was calculated as described (*11*). Control cells showed 15% spontaneous DNA fragmentation in serum-free media. All results are in duplicate and represent at least three separate experiments.
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TECHNICAL COMMENTS

Ambiguities in Ab Initio Phasing

One of the main reasons that determining macromolecular crystal structures is difficult is the absence of phase information for the observed diffraction intensities or amplitudes. This phase problem is often overcome by exhaustive preparation and analysis of heavy atom derivatives of a crystallized molecule. Diffraction measurements on crystals in which heavy atoms have bound uniformly to a macromolecule, when taken with data from native crystals, can lead to approximate values for the missing phases.

Direct methods for determining the phases of a small molecule structures without the use of additional experimental data have been developed and are now the main method for obtaining phases for crystals of these molecules (*1*). Interest in similar ab initio phasing approaches for macromolecules has been great, but progress has been slow (*2*). A significant step toward ab initio phasing, in the absence of any experimental phase information, would be the direct determination of the protein-solvent boundary from diffraction intensities. S. Subbiah (*3*) describes an algorithm designed to produce such an envelope by modeling the contents of the unit cell with a hard-sphere gas, which maximizes the correlation between observed and calculated structure factor amplitudes. Although this idea seems feasible, the analysis by