subsequently become quiescent because of IL-2 receptor down-regulation (*18*). These cells can be stimulated to reenter the cell cycle by treatment with PHA and PMA yet have a better-defined morphology than PBT cells. Quiescent cells showed faint cytoplasmic staining with affinity-purified anti–GST-PAC (Fig. 4B). Cells stimulated for 1 hour demonstrated slightly more intense staining that was mostly in the cytoplasm but could be distinguished also in the nucleus. Four hours after stimulation, intense staining in the nucleus was observed in the majority of cells, although heterogeneity was apparent. Eight hours after stimulation, the amount of protein had dropped to the amount present before stimulation, and the protein appeared again in both the cytoplasm and the nucleus.

A nuclear tyrosine phosphatase might act on any number of potentially important substrates, including phosphoproteins that regulate cell cycle progression or transcription. It recently has been demonstrated that tyrosine phosphorylation regulates the localization and activity of the transcription factor complex that mediates interferon-α–induced gene expression (*19*). Tyrosine phosphorylation and dephosphorylation of nuclear proteins may be a generalized mechanism for signal transduction stimulated by other ligands or physiological situations.

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Requirement for Cdk2 in Cytostatic Factor–Mediated Metaphase II Arrest

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The unfertilized eggs of vertebrates are arrested in metaphase of meiosis II because of the activity of cytostatic factor (CSF). *Xenopus* CSF is thought to contain the product of the *Mos* proto-oncogene, but other proteins synthesized during meiosis II are also required for arrest induced by CSF. In *Xenopus* oocytes, ablation of synthesis of cyclin-dependent kinase 2 (Cdk2) during meiosis resulted in absence of the metaphase II block, even though the Mos^{xe} protein kinase was fully active at metaphase. Introduction of purified Cdk2 restored metaphase II arrest, and increasing the amount of Cdk2 during meiosis I (when Mos^{xe} is present) led to metaphase arrest at meiosis I. These data indicate that metaphase arrest is a result of cooperation between a proto-oncogene kinase and a cyclin-dependent kinase and illustrate the interaction of a cell growth regulator with a cell cycle control element.

Recent progress in the study of cell cycle control has established that the regulation of both meiosis and mitosis (M phase) in eucaryotic cells is controlled by the activity of the Cdc2 protein kinase (*1*). When active, Cdc2 is complexed with a specific form of cyclin molecule, and cell cycle transitions from M phase to interphase are characterized by degradation of the associated cyclin component and inactivation of Cdc2 protein kinase activity (*2*). A different form of M phase control is evident in meiosis II in vertebrates, when the cell cycle becomes arrested in metaphase in the presence of high amounts of Cdc2 activity and maximal amounts of cyclin B. Microinjection of cytoplasm from metaphase II–arrested, unfertilized frog eggs into blastomeres of fertilized eggs causes metaphase arrest (*3*). The name given to this activity is cytostatic factor (CSF). Little is known about the biochemical nature of CSF, but it is stabilized by Mg^{2+}-adenosine triphosphate, NaF, and EGTA (*3*). The product of the *Xenopus* c-*Mos* proto-oncogene (c-Mos^{xe}), a serine-threonine protein kinase, also causes metaphase arrest in embryonic blastomeres, and moreover, immunodepletion of c-Mos^{xe} from extracts of unfertilized eggs depletes CSF activity (*4*). These results suggested that c-Mos^{xe} could be a component of CSF. Upon fertilization of eggs, the intracellular concentration of free Ca^{2+} increases, cyclin is rapidly degraded, followed, more slowly, by the disappearance of the c-Mos^{xe} protein that migrates with a slower electrophoretic mobility (*5*). These results suggest that the loss of CSF activity at fertilization involves changes in the expression of c-Mos^{xe}.

Protein synthesis is required for both meiosis I and meiosis II in *Xenopus* and mice (*6*), and the ability of recombinant c-Mos^{xe} to induce meiosis I in *Xenopus* oocytes has been taken as evidence that the sole protein synthesis requirement for meiosis I is accounted for by the new synthesis of c-Mos^{xe} (*7*). However, meiosis II in *Xenopus* is not stimulated by the active c-Mos^{xe} kinase, suggesting that other newly synthesized components are also required (*7*). One possible component that could account for the additional protein synthesis requirement in meiosis II is the product of the cyclin-dependent kinase 2 gene (*Cdk2*) in *Xenopus*. The mRNA encoding Cdk2 is newly polyadenylated in oocytes stimulated to enter meiosis I by addition of progesterone, and after fertilization, the Cdk2 mRNA is deadenylated, suggesting that, like c-Mos^{xe}, the Cdk2 protein kinase functions in the meiotic cell cycles of oocytes (*8*).

In *Xenopus* embryos Cdk2 protein kinase activity is evident only in a complex of large molecular size, and is higher in M phase than in interphase (*9*). The active kinase persists at least until gastrulation (*10*). During oocyte maturation, the activity of Cdk2 is low at germinal vesicle breakdown (GVBD) in meiosis I and increases by meiosis II (*9*). In embryonic or somatic cell cycles Cdk2 may also function in G_1 in regulating entry into S phase (*11*) or in the mechanism coupling activation of

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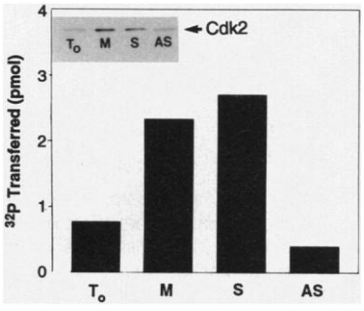

Fig. 1. Ablation of Cdk2 synthesis and H1 kinase activity by antisense oligodeoxynucleotides. Resting oocytes were injected with a mixture of sense or antisense oligodeoxynucleotides to Cdk2 with amidate-modified internucleoside linkages. Two hours later the oocytes were stimulated to mature by addition of progesterone. Samples of 15 oocytes were taken 8 hours after addition of progesterone. Cdk2 was immunoprecipitated, and H1 kinase activity in immune complexes was assayed as described (*9, 15*). T_o, resting oocytes; M, mock-injected; S, sense-injected; AS, antisense-injected oocytes. Inset: Samples were immunoblotted for Cdk2.

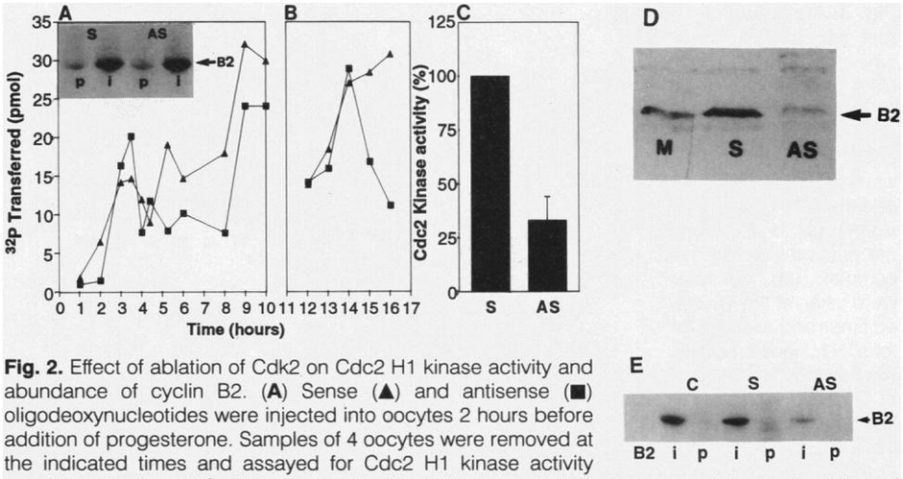

Fig. 2. Effect of ablation of Cdk2 on Cdc2 H1 kinase activity and abundance of cyclin B2. (**A**) Sense (▲) and antisense (■) oligodeoxynucleotides were injected into oocytes 2 hours before addition of progesterone. Samples of 4 oocytes were removed at the indicated times and assayed for Cdc2 H1 kinase activity precipitated with p13-Sepharose. Inset: c-Mos^{xe} kinase activity was measured in oocytes injected with sense (S) and antisense (AS) oligodeoxynucleotides that were matured to metaphase II with progesterone treatment. Proteins from the oocytes were precipitated with preimmune serum or antiserum to c-Mos^{xe} and assessed for phosphorylation of cyclin B2 in immune complex assays as described (*23*). (**B**) In a separate experiment with different oocytes, H1 kinase activity declined in antisense-injected oocytes after the oocytes reached metaphase of meiosis II, whereas sense-injected oocytes remained arrested with high H1 kinase activity. Only Cdc2 activity in meiosis II is shown (GVBD was at 7 hours). (**C**) Results from 7 different experiments like that in (B). A significant reduction in the Cdc2 H1 kinase activity was seen 2 hours after metaphase II in antisense-injected oocytes as compared to sense-injected controls. (Bar = mean ± SEM; $n = 7$; $p < .001$). (**D**) Immunoblot showing the amount of cyclin B2 2 hours after reaching metaphase II arrest in oocytes injected with sense oligodeoxynucleotides or antisense oligodeoxynucleotides. Results shown are representative of several experiments. (**E**) Kinase activity of c-Mos^{xe} in immune complexes from oocytes injected with sense or antisense oligodeoxynucleotides to Cdk2, 2 hours after reaching metaphase II. p, preimmune serum; i, immune serum; S, sense oligodeoxynucleotides; AS, antisense oligodeoxynucleotides; C, control; B2, cyclin B2 incubated in the absence of serum.

mitosis to completion of DNA replication (*12*). In some somatic cell systems, Cdk2 has been found associated with cyclin A (*13*), but in *Xenopus* eggs, Cdk2 is not bound to cyclin A or any newly synthesized protein (*12*), and its mitotic activation occurs in the presence of protein synthesis inhibitors (*9*).

To investigate the role of Cdk2 in the control of meiosis, several antisense oligodeoxynucleotides to Cdk2 were designed (*14*) and introduced by microinjection into resting oocytes to destroy Cdk2 mRNA prior to polyadenylation. The oocytes were then stimulated to enter into meiosis with progesterone. Antisense oligodeoxynucleotides to Cdk2 ablated both the increased synthesis of Cdk2 during maturation and the increased protein kinase activity associated with Cdk2 (Fig. 1). Histone H1 protein kinase activity (*15*) of Cdc2–cyclin B (maturation promoting factor) was measured during meiosis I and II in the presence of either sense or antisense Cdk2 oligodeoxynucleotides (Fig. 2). Cdc2 H1 kinase activity peaked at GVBD (approximately 3.5 hours after the oocytes were treated with progesterone), declined, and then increased again at metaphase II arrest (8 to 9 hours after progesterone addition). Ablation of Cdk2 had little consistent effect on either the kinetics or magnitude of the oscillatory activity of Cdc2 during meiosis I or meiosis II (Fig. 2). Similar results were obtained with injection of each oligodeoxynucleotide alone. Moreover, the amount of c-Mos^{xe} kinase activity was unaffected by the Cdk2 antisense oligodeoxynucleotides (Fig. 2A), and the overall rate of protein synthesis was also unaffected. However, oocytes that were injected with antisense oligodeoxynucleotides failed to maintain a metaphase arrest after reaching meiosis II and instead exited into the first embryonic cell cycle, whereas control oocytes injected with sense oligodeoxynucleotides remained arrested in meiosis II and had high Cdc2 H1 kinase activity (Fig. 2B). When measured in oocytes 2 hours after the cells reached metaphase II, Cdc2 kinase activity in antisense-injected oocytes was only about 30% of that in oocytes injected with sense oligodeoxynucleotides. The premature exit from metaphase II that was associated with ablation of Cdk2 appeared to represent a normal exit, because cyclin B2 was degraded in antisense-injected oocytes but not in sense-injected or mock-injected oocytes (Fig. 2D). Also, the amount of c-Mos^{xe} kinase activity declined in oocytes injected with antisense oligodeoxynucleotides but

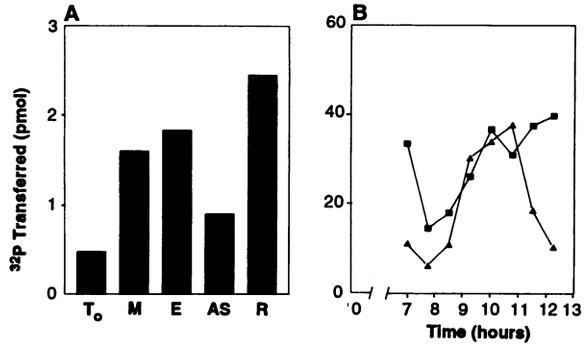

Fig. 3. Metaphase II arrest induced by Cdk2 in antisense-injected oocytes. (**A**) Oocytes were either mock-injected (M) or microinjected with purified Cdk2 monomer (*16*) alone (E), antisense oligodeoxynucleotides (AS), or both purified Cdk2 and the antisense oligodeoxynucleotides (R). Cdk2 H1 kinase activity was measured in immune-complex assays 8 hours after progesterone addition. (**B**) In a different experiment from the one shown in (A), oocytes were injected with antisense oligodeoxynucleotides to Cdk2 in the presence (■) or absence (▲) of purified monomeric Cdk2, and maturation was induced subsequently with progesterone. The amount of Cdk2 injected was sufficient to recover the normal amount of Cdk2 H1 kinase activity when assayed later in metaphase II, as in (A). The H1 kinase activity of Cdc2 during the cell cycle was assayed at the indicated times after progesterone administration. GVBD had occurred at 6 hours and metaphase II was reached at 10 to 11 hours.

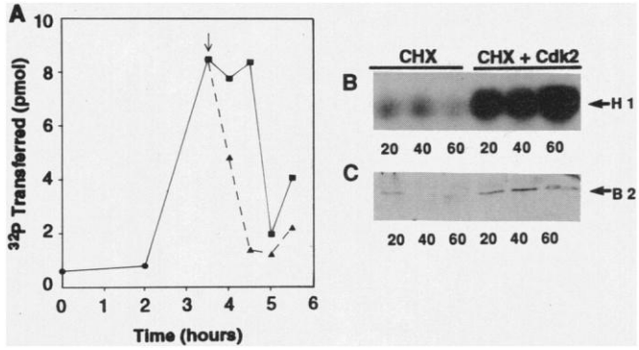

Fig. 4. Metaphase I arrest caused by premature expression of Cdk2. (**A**) Oocytes were treated with progesterone. Three hours later [within 10 min after GVBD (arrow)], they were injected with 50 nl of either CHX alone (100 μg/ml) (▲) or CHX and the purified Cdk2 kinase complex (■). Samples were taken at the indicated times and assayed for total H1 kinase activity. (**B**) Samples from an experiment similar to that in (A) were taken 20, 40, and 60 min after injection of CHX alone or CHX and the purified Cdk2 kinase complex. At the times indicated, Cdc2 was immunoprecipitated with antibody to Cdc2 and assayed for H1 kinase activity in immune-complex assays. H1, histone H1. Direct quantitation of immune-complex assays in injected oocytes showed an activity of 8 pmol per 15 oocytes in 20 min for Cdc2 and 1 pmol per 15 oocytes in 20 min for Cdk2. The latter value is about 60% of that normally present at meiosis II. (**C**) Cyclin B2 (B2) was detected in the same samples as indicated in (B) by immunoblotting.

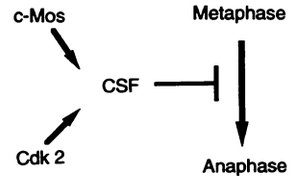

Fig. 5. Model for mechanism of metaphase arrest. The model depicts the arrest of the cell cycle in metaphase by cytostatic factor (CSF) and the requirement for both c-Mos^{xe} kinase and Cdk2 for expression of CSF.

not sense oligodeoxynucleotides (Fig. 2E). These experiments suggest that the activity of Cdk2 is required for maintenance of metaphase II arrest in unfertilized eggs.

To confirm that the effect of the antisense oligodeoxynucleotides is specific, we microinjected purified monomeric Cdk2 (*16*) into oocytes along with the antisense oligodeoxynucleotides and then stimulated entry into meiosis with progesterone. Antisense oligodeoxynucleotides decreased the H1 kinase activity of Cdk2 observed during maturation, but injection of monomeric Cdk2 with the antisense oligodeoxynucleotides resulted in restoration of the Cdk2 activity (Fig. 3A). Cdk2 H1 kinase activity during metaphase II in these oocytes was even greater than that in the controls (Fig. 3A). Injection of purified Cdk2 with the antisense oligodeoxynucleotides restored the metaphase II arrest for several hours by maintaining increased Cdc2 protein kinase activity. In contrast, oocytes injected with only antisense oligodeoxynucleotides exited meiosis II shortly after reaching metaphase II and contained low amounts of Cdc2 protein kinase activity. These experiments indicate that the failure to arrest in metaphase is a specific consequence of the loss of Cdk2 protein kinase activity.

These experiments provide evidence that the c-Mos^{xe} protein kinase and the Cdk2 protein kinase cooperate in the creation of a metaphase II block during meiosis II. During metaphase of meiosis I, an increase in the synthesis of c-Mos^{xe} is required, whereas Cdk2 activity is low (*4*, *9*). Moreover, in the early embryonic cell cycles, c-Mos^{xe} is absent, but Cdk2 activity is increased in M phase (*4*, *5*, *9*, *17*) and reintroduction of c-Mos^{xe} leads to metaphase arrest (*4*, *7*). This situation suggested that expression of both kinases at metaphase in the first meiotic cell cycle should also result in metaphase arrest. To test this possibility, oocytes were treated with progesterone and allowed to progress into meiosis I, a process that requires synthesis and probably phosphorylation of c-Mos^{xe} (*4*). Within 10 min after GVBD, cycloheximide (CHX) was microinjected into the oocytes, and this led rapidly to the loss of Cdc2 H1 kinase activity, which was caused by the rapid degradation of cyclins (*6*, *18*). However, microinjection of purified catalytically active Cdk2 (*19*) into oocytes at the same time as CHX led to a stabilization of the H1 kinase activity of Cdc2 for about 1 hour before it declined (Fig. 4A). Moreover, the increased H1 kinase activity detected in the Cdk2-injected oocytes was associated with enhanced stability of cyclin B2 (Fig. 4C). The bulk of the elevated H1 kinase activity measured in these experiments was due to Cdc2 and not to the introduced Cdk2 (Fig. 4B). This effect appeared to be specific for meiosis I, because injection of Cdk2 into one blastomere of a two-cell embryo did not cause metaphase arrest. The shorter duration of arrest at meiosis I compared to meiosis II could be a consequence of lower amounts of c-Mos^{xe} in meiosis I that might result from faster ubiquitin-mediated protein degradation or changes in phosphorylation of c-Mos^{xe} (*20*). Alternatively, the lower amount of Cdk2 that could be microinjected into cells in meiosis I as compared to that present in unfertilized eggs might lead to a shorter duration of arrest. These results support the concept that the protein kinase activity of both c-Mos^{xe} and Cdk2 is required for metaphase II arrest. The low amount of Cdk2 normally present at meiosis I may prevent metaphase arrest from occurring in M phase even though c-Mos^{xe} is expressed. It appears that c-Mos^{xe} alone causes metaphase arrest in blastomeres because Cdk2 is already active at that stage (*9*).

These results indicate meiotic arrest is a consequence of the synthesis of two protein kinases during oocyte maturation, c-Mos^{xe} and Cdk2. These proteins are not synthesized after fertilization, and although c-Mos^{xe} kinase activity declines rapidly after exit from meiosis II (Fig. 2E), the Cdk2 protein kinase complex remains active at least until gastrulation (*9*, *10*). Each of these kinases may have other specific functions. For example, c-Mos^{xe} is also required for meiosis I whereas Cdk2 is not, and Cdk2 protein kinase activity oscillates in the embryonic cell cycle when no c-Mos^{xe} kinase activity is present. In CSF one kinase component (Cdk2) is cell cycle–regulated while the other (c-Mos^{xe}) is implicated in neoplastic transformation. It is not clear how the transforming activity of c-Mos^{xe} is related to its function in CSF, but evidence suggests that transforming functions are mediated in the G_1 phase of the cell cycle (*21*). Cdk2 also appears to have different functions in M phase and G_1 phase (*9*, *11*), and Cdk2 may also take part in transformation through binding to p107 (*22*). Formally, each kinase could be an essential component of CSF, act upstream to activate CSF, be a target of CSF, or act on independent pathways. Only purification of CSF will resolve these possibilities. However, we favor the concept that both kinases are essential components of CSF (Fig. 5).

The mechanism underlying the dual action of c-Mos^{xe} and Cdk2 is not known at present. One possibility is that both kinases phosphorylate a common substrate required for metaphase arrest. Possible candidates for this substrate include tubulin and cyclin B2 (*23*). Cyclin B2 is a substrate for c-Mos^{xe} and Cdk2 at sites distinct from those phosphorylated by Cdc2 (*23–25*). The sites in cyclin B2 phosphorylated by Cdc2 are not important for degradation or activation of the Cdc2 H1 kinase activity (*26*), but the effect of phosphorylation by c-Mos^{xe} and Cdk2 has not yet been determined. Another explanation is that two or more separate substrates, each phosphorylated by one or the other kinase, are required for metaphase II arrest. In any case, these results explain

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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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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