The Cyclin-dependent Kinase Cdk2 Regulates Thymocyte Apoptosis

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Summary

Aberrant activation of cell cycle molecules has been postulated to play a role in apoptosis ("catastrophic cell cycle"). Here we show that in noncycling developing thymocytes, the cyclin-dependent kinase Cdk2 is activated in response to all specific and nonspecific apoptotic stimuli tested, including peptide-specific thymocyte apoptosis. Cdk2 was found to function upstream of the tumor suppressor p53, transactivation of the death promoter Bax, alterations of mitochondrial permeability, Bcl-2, caspase activation, and caspase-dependent proteolytic cleavage of the retinoblastoma protein. Inhibition of Cdk2 completely protected thymocytes from apoptosis, mitochondrial changes, and caspase activation. These data provide the first evidence that Cdk2 activity is crucial for the induction of thymocyte apoptosis.

Key words: cyclin-dependent kinase 2 • apoptosis • cell cycle • thymocyte

Apoptosis or programmed cell death (PCD) is required for all multicellular organisms to maintain the homeostasis of their organ systems. Failure to invoke effective programmed cell death can result in developmental abnormalities, cancer, or autoimmune diseases, whereas increased apoptosis produces degenerative diseases of the brain or immunodeficiencies (1). Apoptosis can be triggered by numerous different stimuli, all of which converge at the common checkpoint of mitochondrially regulated death induction (1–4) and caspase activation (5–9).

Apoptosis and mitosis have many features in common, including cytoskeletal changes, nuclear envelope breakdown, and chromatin condensation, and it has been speculated that apoptosis may result from a form of aberrant cell cycling called "catastrophic mitosis" (10–12). This idea is supported by the fact that various gene products that have marked effects on cell cycle control, such as p53, retinoblastoma protein (Rb), 1 Cdc25, M ax, c-M yc, or E2F-1, also regulate susceptibility to apoptosis (13–20). Thus, it has been shown that overexpression of the tumor suppressor p53 can induce either growth arrest or apoptosis, depending on the cell type (21). Conversely, loss of p53 function in mice produces resistance to apoptotic stimuli such as γ-irradiation (22, 23). Furthermore, deficiency of another cell cycle regulator, E2F-1, in mice resulted in an enlarged thymus in these mutant mice, implying a possible role of E2F-1 as a proapoptotic molecule (19, 20). Inversely, the loss of the tumor suppressor and cell cycle regulator Rb in mice leads to increased cell death, further confirming the close interaction between apoptotic pathways and cell cycle pathways (24).

Members of the cyclin-dependent kinase (Cdk) family of serine/threonine kinases are known to be key regulators of eukaryotic cell cycle progression (25). Different Cdk catalytic subunits and their activating cyclin subunits operate as control checkpoints during cell cycle progression. Cdk2 is crucial for the progression from the G1 to the S phase of the cell cycle. Inhibition of Cdk2 activity in vitro has been shown to protect cultured sympathetic neurons and heart muscle cells from apoptosis (26, 27). However, Cdk2 inhibition can also lead to cell death in tumor cell lines (28). Whether Cdk2 and the aberrant activation of the cell cycle machinery have an apoptotic function necessary for normal development has yet to be addressed.

CD4+CD8+ thymocytes are noncycling cells which are sensitive to many apoptotic stimuli in vitro and in vivo, including glucocorticoids, ionizing irradiation, heat shock, and CD95 (9). Physiologically, CD4+CD8+ thymocytes undergo negative selection and clonal deletion required for the induction and maintenance of immunological tolerance (29). We report in developing thymocytes that Cdk2 is activated in response to all specific and nonspecific apoptotic stimuli tested, including γ-irradiation and peptide-specific...
negative thymocyte selection. Inhibition of Cdk2 completely prevented all aspects of thymocyte apoptosis and blocks peptide-specific thymocyte death.

**Materials and Methods**

Mice. p53 gene–deficient, Bcl-2 transgenic (Tg), DO.11.10 OVA TCR-α/β Tg, and P14 TCR-α/β Tg mice have been described previously (30–33). BALB/c mice were purchased from Taconic Farms. All mice were kept at the Animal Facility of the Ontario Cancer Institute in accordance with institutional guidelines.

Apoptosis Induction and Inhibitors. Freshly isolated thymocytes from BALB/c mice were cultured in RPMI 1640 medium (10% FCS, 10^{-3} M β-mercaptoethanol) in the absence or presence of dexamethasone (Sigma), heat shock, γ-irradiation, anti-CD95 Ab (clone Jo29; PharMingen), anti-CD3ε (clone 145-2C11; Pharmingen), PMMA (12.5 mg/ml), or etoposide (2.5 μg/ml) for different time periods and at different concentrations as indicated in the figure legends (34). Optimal concentrations and activation regimes for the induction of apoptosis were determined in pilot studies. The specific Cdk2 blockers olomoucine (Calbiochem) and roscovitine (gift of Dr. M. ejer, CNRS, Roscoff, France) were dissolved in DM SO (Sigma). Titration experiments determined that 100 μM olomoucine and 50 μM roscovitine were the most effective concentrations for inhibiting Cdk2 activity and apoptosis in thymocyte cultures. DM SO had no effect on Cdk2 activity or the induction or prevention of apoptosis in response to all stimuli tested. Th cell cycle blockers TGF-β (1 nM; R&D Systems) and rapamycin (2 ng/ml; Calbiochem) were used at concentrations that optimally blocked cell cycle progression in T cell lymphoma cells.

Detection of Apoptosis. Total cell numbers of viable and apoptotic cells were determined by trypan blue exclusion. Relative percentages of viable and apoptotic CD4^{+} CD8^{−} thymocytes were determined by triple staining with anti-CD4-PE, anti-CD8-FITC, and the vital chromogenic dye, 7AAD (34). The results were expressed as the percentage of viable thymocytes remaining after 22 h, calculated as follows (number of viable CD4^{+} CD8^{−} thymocytes after stimulation)/(number of viable CD4^{+} CD8^{−} thymocytes cultured under the same conditions in the absence of stimulation) × 100. For the detection of cycling and apoptotic cells, thymocytes were stained with propidium iodide (PI). After different periods of stimulation, thymocytes were harvested, washed once in PBS (0.5% glucose), and fixed in cold 70% ethanol overnight. Fixed cells were pelleted to remove ethanol and stained with PI (final concentration 50 μmol/ml) for 30 min at room temperature. Apoptosis–mediated membrane changes were determined via staining with Annexin V (R&D Systems), PI and Annexin V staining of thymocytes was determined by flow cytometry using a FACSCalibur™ (Becton Dickinson).

Kinesin Assays. After different periods of stimulation, thymocytes were harvested and lysed, and proteins were immunoprecipitated using Abs against Cdk2 (amino acids [aa] 283–298), Cdk4 (aa 282–303), and Cdc2 (aa 278–297) (all from Santa Cruz Biotechnology). Cdk2 and Cdc2 kinase activities in immunoprecipitates were assayed using [γ-32P]ATP (3,000 cpm/ml) and histone H1 (2 μg/ml; Boehringer Mannheim) or p53 (2 μg/ml; Pharmingen) as substrates. Cdk4 activity was determined using glutathione S-transferase (GST)-Rb as a substrate (2 μg/ml; Pharmingen). H1, p53, or GST-Rb phosphorylation was assayed by autoradiography after SDS-PAGE separation. The levels of immunoprecipitated Cdk2, Cdc2, and Cdk4 were determined by Coomassie blue staining and Western blotting.

Immunoprecipitations and Western Blotting. Thymocytes were lysed in 1% NP-40 lysis buffer. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with Abs reactive to Cdc2, Cdk2, Cdk4, Cdc7, Ptaire-2, Cdc25A, cyclins A, E, D1, D2, B, and D3, E2F-1, p27^{kip}, caspase 2, and p53 (clone 240) (all from Santa Cruz Biotechnology), p21 (Calbiochem), Bcl-X L (Transduction Laboratories), Bcl-2 (Pharmingen), caspase 3 (gift of Dr. R. Sekaly, McGill University, Montreal, Quebec, Canada), and Rb (clone G3-245 reactive to an aa 300–380 epitope of Rb, Pharmingen; and clone C-15 reactive against aa 914–928, Santa Cruz Biotechnology). The anti-caspase S-specific Abs were developed in our Institute and were a kind gift of Dr. R. Hakem (Amgen Institute). Immunoprecipitations were performed using protein A-Sepharose. Optimal Ab concentrations and conditions for immunoprecipitations were determined in pilot studies.

Δψ_{m} Disruption. The mitochondrial transmembrane potential (Δψ_{m}) results from the asymmetric distribution of protons across the inner mitochondrial membrane, giving rise to a chemical (pH) and electric gradient (35, 36). The inner side of the inner mitochondrial membrane is negatively charged. As a consequence, the catonic lipophilic fluorochrome 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) is distributed on the mitochondrial matrix as a function of the Nernst equation, correlating with Δψ_{m}. DiOC6(3) can be used to measure variations in the Δψ_{m} on a per-cell basis. Cells induced to undergo apoptosis manifest an early reduction in the incorporation of Δψ_{m}–sensitive dyes, indicating a disruption of Δψ_{m}. For DiOC6(3) staining, 10^{6} thymocytes were incubated with DiOC6(3) (final concentration 20 nM in PBS) for 20 min at 37°C. DiOC6(3) staining was analyzed immediately using a FACSCalibur™.

In Vitro Negative Thymocyte Selection. Thymocytes were purified from P14 Tg mice, which express an α/β TCR (TCR αβVβ8) specific for a peptide (p33) of the lympholytic chorioneviruses virus (LCMV V). P14 Tg thymocytes (10^{6}/well) were cultured on a monolayer of confluent and adherent M C57/L fibroblasts (H-2^{b}) in RPMI 1 medium (5% FCS, 10^{-5} M β-mercaptoethanol). M C57/L APCs were pulsed with different concentrations of the deleting LCMV-p33 peptide for 2 h before coculture with thymocytes. Thymocytes were harvested after 22 h incubation and stained with anti-CD4-PE, anti-CD8-FITC, and the dye, 7AAD. Percent survival was calculated as follows: (total number of viable CD4^{+} CD8^{−} thymocytes cultured at different LCMV-p33 peptide concentrations)/(total number of viable CD4^{+} CD8^{−} thymocytes cultured with M C57/L cells at 37°C in the absence of peptide) × 100. Total numbers of viable and dead cells were determined by trypan blue exclusion. For detection of Cdk2 activity in peptide-activated thymocytes, P14 Tg thymocytes (10^{6}/well) were cultured on a monolayer of confluent (noncytolytic) and adherent M C57/L fibroblasts pulsed with 10^{-5} M of the deleting p33 peptide (37, 38). After 5 h incubation, thymocytes were separated from adherent fibroblasts and subjected to Cdk2 kinase assays as above.

Fetal Thymic Organ Culture. DO.11.10 males were mated with estrous BALB/c females. On day 16 of gestation, pregnant females were killed and embryonic thymi were harvested and placed in culture. Thymi were microdissected and placed on the surface of 0.8-mm filters (Nucleopore) resting on Gelfoam gela-
protein was added at 1 mg/ml on day 1 of culture, and thymi were analyzed 20 and 40 h later. Cell numbers were determined by counting in the presence of trypan blue.

**Results**

Cdk2 Kinase Activity in Thymocyte Apoptosis. To determine whether the cell cycle machinery has a role in apoptosis of noncycling G1 CD4+CD8+ thymocytes, we analyzed the activity of several Cdns in these cells. Freshly isolated thymocytes were treated with different death stimuli such as dexamethasone, heat shock, γ-irradiation, and CD95 for different time points, and Cdk activities of stimulated as well as control thymocytes were assessed in in vitro kinase assays. Surprisingly, increased activity of the cyclin-dependent kinase Cdk2 was detected within 30 min of dexamethasone activation and peaked at 5 h (Fig. 1 A). Cdk2 activity was rapidly increased in response to all apoptotic stimuli tested, including dexamethasone, anti-Fas (CD95) cross-linking, heat shock, or γ-irradiation (Fig. 1 B). No changes in the kinase activities of Cdk4 or Cdc2 were observed after induction of apoptosis (Fig. 1 A and C). Cdc2 activation was also not observed using an anti-Cdc2 phosphorylation epitope-specific Ab indicative of Cdc2 activation. Cdc2 and Cdk4 activities were readily detectable in cycling T lymphoma cells (not shown). The expression levels of molecules involved in the cell cycle, such as Cdk2, Cdk4, Cdc2, Cdk7, Pctaire-2, Cdc25A, cyclins A, B, D1, D2, D3, and E, and E2F-1, did not change 5 h after stimuli (not shown). Cdk2 was found to bind to cyclin A and E thymocytes after treatment with dexamethasone and γ-irradiation. Immunoprecipitations of both cyclin A and cyclin E showed that after γ-irradiation or dexamethasone, histone H1 was phosphorylated, suggesting that Cdk2 was activated.

**Figure 1.** Induction of Cdk2 kinase activity in response to apoptotic stimuli. (A) Kinetics of Cdk2 and Cdc2 activity after dexamethasone treatment. Freshly isolated thymocytes were cultured in the absence or presence of dexamethasone (1 μM), and Cdk2 and Cdc2 activities were assessed. After the indicated periods of stimulation, cells were harvested and lysed, and proteins were immunoprecipitated using Abs against Cdk2 and Cdc2. Cdk2 and Cdc2 kinase activities in immunoprecipitates were assayed using [γ-32P]ATP and histone H1 as the substrate. One result representative of four independent experiments is shown. (B) Induction of Cdk2 activity in thymocytes in response to dexamethasone (1 μM), heat shock (42°C for 1 h), anti-CD95 (1 μg/ml), and γ-irradiation (500 rads). Thymocytes were harvested 5 h after stimulation. Top panel, histone H1 phosphorylation; middle panel, level of Cdk2 protein; bottom panel, kinase activity measured in arbitrary units normalized to background protein and kinase levels. One result representative of five independent experiments is shown. (C) Activity of Cdc2 and Cdk4 after induction of apoptosis. Thymocytes were cultured with dexamethasone (Dex) to induce apoptosis. After 5 h, cells were lysed and Cdc2 and Cdk2 were immunoprecipitated. Cdc2 and Cdk4 kinase activities were determined using histone H1 and GST-Rb as substrates, respectively. (D) Blockage of Cdk2 activity in thymocytes by specific inhibitor roscovitine (50 μM) after dexamethasone (Dex) treatment. Similar results were obtained using the Cdk2 blocker olomoucine. Note the background kinase activity of Cdk2 in A, B, and D which is due to the spontaneous apoptosis of thymocytes in culture (∼20% of thymocytes die within 24 h by default). Blockage of Cdk2 inhibits this form of default death and even after 1 wk in culture, >90% of thymocytes were viable in the presence of roscovitine or olomoucine (not shown). (E) Activity of Cdk2–cyclin A or Cdk2–cyclin E complexes on histone H1 after dexamethasone (1 μM) treatment. Cyclin A or cyclin E was immunoprecipitated using specific Abs, and kinase assays were performed as in A.
ing that both cyclin A and cyclin E have a role in Cdk2 activation (Fig. 1 E). These results show that the induction of thymocyte apoptosis by dexamethasone, γ-irradiation, heat shock, or CD95 cross-linking leads to the activation of the cell cycle regulator Cdk2.

Inhibition of Cdk2 Blocks Thymocyte Apoptosis. To test whether Cdk2 activity was required for the induction of thymocyte apoptosis, the effects of two specific inhibitors of Cdk2, olomoucine and roscovitine (39, 40), were examined. These inhibitors are purine analogues that selectively inhibit the activity of Cdk2 and Cdc2 by specific binding to the ATP-binding pocket. Both molecules completely inhibited dexamethasone-induced Cdk2 activation in thymocytes (Fig. 1 D), and blocked thymocyte apoptosis after stimulation with dexamethasone, heat shock, γ-irradiation, PMA, or the DNA damaging agent, etoposide (Fig. 2 A). PI staining confirmed that thymocytes were in the G1 phase of the cell cycle and that induction of apoptosis did not correlate with cell cycle progression (Fig. 2 D). A “point of no return” was reached between 15 and 30 min after treatment with the apoptotic stimulus such that a Cdk2 blocker added after this time was unable to prevent apoptosis (Fig. 2 B). Addition of other cell cycle blockers such as TGF-β1 or rapamycin, used at the optimal concentrations, had no effect on the kinetics or extent of thymocyte death (Fig. 2 C). Interestingly, although CD95 cross-linking led to strong Cdk2 activation (Fig. 1 B), Cdk2 blockers did not inhibit CD95-mediated thymocyte death (Fig. 2 A). In fact, CD95-mediated apoptosis was consistently enhanced in the presence of the Cdk2 blockers. These results imply that CD95 uses a pathway other than the one used by the other inducers, i.e., a receptor/caspase 8 pathway instead of a nucleus/mitochondrial/caspase 9 pathway (41). It should be noted that in our screen, CD95 activation is the only thymocyte death stimulus so far that cannot be blocked by Cdk2 inhibition.

Cdk2 Kinase Activity Is Required for Peptide-specific Thymocyte Apoptosis. The process of clonal deletion and selection-triggered thymocyte death is a fundamental mechanism required for the maintenance of lymphocyte homeostasis and immunotolerance. CD4+CD8+ thymocytes expressing TCRs which recognize self-antigens with high affinity/
avidity are clonally deleted via apoptosis, leading to the removal of T cells that express TCRs with potentially harmful self-reactivity (thymic tolerance [29]). To test whether Cdk2 is a physiological regulator of thymocyte apoptosis, we induced apoptosis of CD4⁺CD8⁺ immature thymocytes by anti-CD3 cross-linking (42). Fig. 2 A shows that Cdk2 inhibitors were able to block anti-CD3-mediated apoptosis of immature thymocytes.

To further investigate the role of Cdk2 in clonal deletion, we used an in vitro negative selection system using thymocytes from P14 Tg mice. P14 Tg mice express a rearranged TCR, which is reactive to the p33 peptide of LCMV. P14 Tg CD4⁺CD8⁺ thymocytes undergo apoptosis after culture with APCs pulsed with different concentrations of the deleting p33 peptide. Thymocytes from P14 Tg mice underwent apoptosis in a p33 peptide dose-dependent fashion which was inhibited by the addition of Cdk2 blockers (Fig. 3 A). Importantly, induction of peptide-specific apoptosis of P14 Tg thymocytes triggered Cdk2 kinase activity (Fig. 3 B). Inhibition of Cdk2 did not interfere with TCR-mediated proximal signaling events or with TCR internalization, which is a functional measure of antigen receptor-mediated activation (not shown). Moreover, inhibition of Cdk2 by olomoucine blocked OVA-mediated negative selection of CD4⁺CD8⁺ OVA-specific TCR Tg thymocytes in fetal thymic organ cultures (FTOCs, Fig. 3 C). Cdk2 blockers did not interfere with positive thymocyte selection in reaggregation culture assays (not shown), indicating that Cdk2 has a specific role in peptide-specific thymocyte apoptosis.

Cdk2 Acts Upstream of Mitochondrial Permeability Transition, Bcl-2, and Caspases. Where does Cdk2 function in the hierarchy of apoptosis? Disruption of DCm due to the opening of mitochondrial pores has been invariably associ-

Figure 3. Cdk2 kinase is activated in peptide-specific thymocyte apoptosis. (A) Inhibition of Cdk2 blocks induction of peptide-specific apoptosis of P14 Tg thymocytes, which express an α/β TCR (TCR α2Vβ8) specific for p33 peptide of LCMV. P14 Tg thymocytes were cultured on p33 glycoprotein peptide-pulsed MC57/L fibroblasts in the absence (Peptide) or presence of roscovitine (50 μM; Peptide+Cdk2-inhibitor). Thymocytes were harvested after 22 h incubation and stained with anti-CD4-PE and anti-CD8-FITC. Percent survival was calculated as follows: (total number of viable CD4⁺CD8⁺ thymocytes cultured at a given concentration of p33 peptide)/(total number of viable CD4⁺CD8⁺ thymocytes cultured with MC57/L cells at 37°C in the absence of peptide) × 100. Clarity of peptide concentrations is shown on the x-axis. Cells were harvested after 22 h of culture, and cell death was determined. Numbers are the percentages of viable CD4⁺CD8⁺ thymocytes cultured at various p33 peptide concentrations compared with control thymocytes in the absence of p33 peptide (Control). Apoptosis of P14 thymocytes in the presence of roscovitine alone is also shown (Cdk2-inhibitor). Similar results were obtained using olomoucine (not shown). One result representative of five independent experiments is shown. (B) Induction of Cdk2 kinase activity by peptide-specific negative selection. P14 Tg thymocytes (10⁷/well) were cultured on a monolayer of adherent MC57/L fibroblasts (H-2 b/b) in the presence or absence of the deleting p33 peptide. After 5 h of culture, thymocytes were harvested. Cdk2 was immunoprecipitated, and Cdk2 activity was assessed using histone H1 as substrate. (C) Cdk2 inhibitors prevent OVA protein (Ova-protein)-induced apoptosis of OVA-specific TCR Tg thymocytes in FTOCs. Thymi were removed at embryonic day 16 and cultured with olomoucine (100 μM) and Ova protein (1 mg/ml). Percentages of viable thymocytes were determined.
ated with apoptosis and is an early common denominator of cell death (43). Alterations in mitochondria lead to the release through the outer mitochondrial membrane of molecules such as cytochrome c and the apoptosis-inducing factor (AIF), and the activation of the caspase cascade (7, 35, 36, 44–48).

To assess whether Cdk2 acts upstream or downstream of mitochondrial events, we examined changes in ΔΨm using cytometry and the fluorochromic dye, DiOC6(3). Thy-mocytes were stimulated either with dexamethasone or anti-CD95, and the mitochondria changes of ΔΨm were assessed at different time points. The first changes in thymocyte ΔΨm were observed 2 h after dexamethasone treatment, and ΔΨm was significantly disrupted after 5 h (Fig. 4A). Addition of Cdk2 inhibitors blocked dexamethasone-induced losses of ΔΨm (Fig. 4A). CD95-mediated ΔΨm disruption and apoptosis still occurred in the presence of Cdk2 inhibitors (Figs. 2A and 4A), implying that Cdk2 inhibition per se does not interfere with opening of mitochondrial pores. Since ΔΨm is regulated by Bcl-2 family members (43, 49), we also tested Cdk2 activation in Bcl-2 Tg thymocytes (31, 50). Although overexpression of Bcl-2 protected thymocytes from dexamethasone- and irradiation-induced cell death and disruption of ΔΨm (31, 50), Cdk2 was still activated in Bcl-2 Tg thymocytes in response to these apoptotic stimuli (not shown).

Caspase activation is a crucial event in apoptosis, and caspases can function upstream or downstream of mitochondrial ΔΨm disruption (8, 9). To determine where Cdk2 acts during apoptosis with regard to the caspase activation cascade, processing of different caspases was assessed in thymocytes after treatment with different apoptotic stimuli in the presence or absence of Cdk2 inhibitors. Within 2 h after dexamethasone and γ-irradiation, caspase 3 (Cpp32) and caspase 8 activation was observed in thymocytes whereas caspase 2 (nedd2) processing was first observed 3 h after death induction. Caspase activation peaked at 5 h after induction of cell death (Fig. 4B, and data not shown).

Figure 4. Cdk2 acts upstream of mitochondrial permeability transition, caspase activation, and Rb cleavage. (A) Mitochondrial permeability transition (ΔΨm disruption). Thymocytes were cultured for 5 h in medium alone, or in medium containing dexamethasone (1 μM) or anti-CD95 (1 μg/ml) in the presence or absence of roscovitine (50 μM). Cells induced to undergo apoptosis manifest an early reduction in the incorporation of ΔΨm-sensitive dyes, indicating a disruption of ΔΨm. For DiOC6(3) staining, 10^6 thymocytes were incubated with DiOC6(3) (final concentration 20 nM in PBS) for 20 min at 37°C. DiOC6(3) staining was analyzed immediately using a FACSCalibur™ flow cytometer. One result representative of three independent experiments is shown. (B) Thymocytes were stimulated with the indicated death stimuli for 5 h, and proteolytic activation of caspase 3 (Cpp32) was assessed by Western blotting. The anti–caspase 3 Ab recognizes both the intact caspase 3 molecule (procaspase 3) and its cleaved 17-kD active form (p17). Addition of roscovitine inhibits caspase 3 cleavage in response to dexamethasone (right) and in response to all other death stimuli tested, except for anti-CD95. (C) Thymocytes were stimulated with the indicated stimuli, and the proteolytic activation of caspase 2 (Nedd2) was assessed by Western blotting. The addition of roscovitine (Rosco) after irradiation blocked caspase 2 cleavage into a p14 fragment. (D) The retinoblastoma protein Rb is proteolytically processed in response to all cell death stimuli. Inhibition of Cdk2 blocked Rb cleavage. Thymocytes were stimulated with dexamethasone and γ-irradiation in the presence or absence of roscovitine (50 μM). After 5 h, cells were harvested and lysed, and the status of Rb was assessed by Western blotting. Rb-reactive Ab recognizes aa 300–380 of Rb. As previously described in cell lines (reference 53), Rb was cleaved of the COOH-terminal end in apoptotic thymocytes in response to all apoptotic stimuli.
However, activation of caspase 3 (Fig. 4 B), caspase 2 (Fig. 4 C), or caspase 8 (not shown) did not occur after blocking of Cdk2 kinase activity. These results show that Cdk2 acts upstream of Bcl-2, ΔF_m, and caspas.

Proteolytic Processing of the Retinoblastoma Protein in Thymocyte Apoptosis. During apoptosis, various cell cycle regulatory molecules such as p21 and Rb are proteolytically cleaved by caspases. In particular, proteolytic processing of the G1 to S cell cycle gatekeeper Rb (ΔR b) has been previously reported in T NF- and C D95-treated tumor cell lines (51, 52). R b and Cdk2 were found to be colocalized in developing thymocytes (not shown). Induction of thymocyte apoptosis in response to dexamethasone, irradiation, heat shock, or anti-CD95 correlated with the appearance of a second smaller R b protein (ΔR b; Fig. 4 D). Although it has been shown that R b is cleaved by caspase 3 (53) and in thymocytes R b was found to be a proteolytic cleavage product of R b mediated by caspases, ΔR b was still observed in caspase 3 gene-deficient mice (not shown). The earliest detectable R b cleavage (ΔR b) occurred 5 h after dexamethasone treatment (Fig. 4 D). Cdk2 inhibitors or transgenic overexpression of Bcl-2 in thymocytes prevented cleavage of R b in response to dexamethasone (Fig. 4 D, and data not shown). These results demonstrate that Cdk2 acts upstream of mitochondrial pore opening, Bcl-2, caspase activation, and proteolytic cleavage of the cell cycle regulator R b. The functional consequences of R b cleavage are not known. Since ΔR b can only be observed downstream of the caspase effector phase and still binds to Cdk2 (not shown), the generation of ΔR b might function as a regulatory feedback loop that could influence Cdk2 and/or E2F-1 activity.

Cdk2 Is Upstream of p53 and Bax Expression in Irradiated Thymocytes. How is Cdk2 activity mechanistically linked to apoptotic mitochondrial events? Various members of the Bcl-2 family of mitochondrial gatekeepers are phosphorylated on serine/threonine residues (9, 49). Although Bcl-2 and Bcl-XL contain consensus sites for Cdk2 activity, we could not detect Cdk2-mediated phosphorylation of either Bcl-2 or Bcl-XL in vitro (not shown). The tumor suppressor p53 is a substrate for Cdk2 in the DNA repair response (54), and p53 transactivation or phosphorylation resistant to γ-irradiation-induced apoptosis but still susceptible to dexamethasone and antigen receptor-mediated cell death (22, 23). The effect of the p53 mutation has been mapped upstream of apoptotic mitochondrial events (55). Therefore, we tested whether p53 is a target for Cdk2 activity during thymocyte apoptosis after γ-irradiation. In vitro kinase assays using immunoprecipitated Cdk2 from γ-irradiated and dexamethasone-treated thymocytes showed that Cdk2 can phosphorylate p53 (Fig. 5 A). Moreover, p53 was found to interact with Cdk2 in thymocytes (Fig. 5 B). To test the effect of Cdk2 activity on p53 expression, we analyzed the levels of p53 protein in γ-irradiated thymocytes in the presence or absence of Cdk2 inhibitors. Although p53 protein accumulated to significant levels after treatment of cells with γ-irradiation alone, little p53 accumulation was observed when cells were treated with γ-irra-
increased in thymocytes after dexamethasone and γ-irradiation, it has been shown in p53 gene-deficient mice that p53 protects thymocytes only from DNA damage, and not from dexamethasone or antigen receptor-mediated cell death (22, 23), implying that other downstream molecules exist that link Cdk2 to apoptosis. Preliminary evidence from our laboratory implies that the glucocorticoid receptor which is required for dexamethasone-mediated cell death can be phosphorylated by activated Cdk2 and coimmunoprecipitates with Cdk2 in dying thymocytes. Besides association with the ligand, phosphorylation of the glucocorticoid receptor is required for its translocation from the cytoplasm into the nucleus (60–62). In addition to the glucocorticoid receptor, other orphan steroid receptors such as Nur77 might be molecular targets for Cdk2 kinase activity.

It has been shown that mitochondria are early checkpoints that integrate multiple death signaling pathways into a common Ced4/caspase-regulated effector mechanism. Opening of mitochondrial pores, mitochondrial swelling, disruption of ΔΨm, and release of proapoptotic molecules, including cytochrome c and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane spaces into the cytoplasm have all been implicated as fundamental mechanisms that initiate and propel the effector phase of apoptosis. Posttranslational modification and the balance between death suppressors, such as Bcl-2 and Bcl-XL, and death promoters, including Bax and Bad, are crucial mechanisms of mitochondrial integrity and the apoptotic effector phase (9, 49). Our results show that Cdk2 acts upstream of ΔΨm disruption, Bax and Bcl-2, and caspase activation in developing thymocytes. Moreover, whereas loss of the mitochondrial transmembrane potential can only be observed 2 h after addition of death stimuli, Cdk2 kinase activity is induced very rapidly and a “point of no return” was reached between 15 and 30 min after treatment with the apoptotic stimulus, such that a Cdk2 blocker added after this time was unable to prevent apoptosis. Thus, our results indicate that Cdk2 is the earliest known common denominator that can integrate many independent apoptotic signals into one common effector pathway.

Although CD95 (Fas) stimulation induced Cdk2 activity in thymocytes, inhibition of Cdk2 did not block CD95-mediated apoptosis. In fact, Cdk2 inhibition enhanced the susceptibility to CD95 killing. So far, CD95-mediated apoptosis is the only death signal in thymocytes that does not rely on Cdk2 activation. Although apoptosis in response to γ-irradiation, heat shock, dexamethasone, or peptide-specific negative thymocyte selection requires active transcription of death genes, apoptosis after CD95 killing can occur in the presence of RNA or protein synthesis inhibitors (9).
Moreover, enucleated cells can undergo apoptosis after CD95 activation, suggesting that all components necessary for CD95-mediated apoptosis are present in cells and that CD95 activation can directly trigger the apoptotic machinery. It should be noted that both Cdk2 inhibitors roscovitine and olomoucine do not prevent transcription or translation in noncycling neurons (63) and that inhibition of Cdk2 did not regulate transactivation of the FasL in thymocytes (not shown).

The specific Cdk2 blockers olomoucine and roscovitine are purine analogues that inhibit Cdk2 kinase activity by specifically binding to the Cdk2 ATP-binding site (39, 40). At concentrations at which olomoucine and roscovitine blocked apoptosis in in vivo thymocyte cultures, these inhibitors had no effects on in vitro kinase activity of PKCα-ζ, Cdk4, Cdk6, Abi, cAMP P- or GMP P-dependent protein kinases (PKA, PKG), mitogen-activated protein kinase (MAPK; extracellular signal regulatory kinase [ERK]1, ERK2), Src family kinases, glycogen synthase kinase 3 (GSK3), casein kinase, receptor tyrosine kinases, myosin light chain kinase, p38/HOG, or stress-activated protein kinase (SAPK)/c-Jun N terminal kinases (JNKs) (39; and data not shown). Through their unique selectivity for Cdk2, roscovitine and olomoucine provide a unique opportunity to study the role of Cdk2 in thymocyte apoptosis. However, we cannot exclude that roscovitine and olomoucine inhibit a yet unidentified kinase, and our results need to be confirmed using genetic model systems. Thus, genetic systems for inducible and thymocyte-specific inactivation/activation of Cdk2 need to be developed in the future. However, our results showing that all death stimuli lead to Cdk2 kinase activity in thymocytes and that two different Cdk2 kinase inhibitors, but not other inhibitors that block G1 to S progression, inhibit thymocyte apoptosis strongly suggest that Cdk2 is a key kinase involved in thymocyte apoptosis.

Cdk2 is crucial for the progression from the G1 to the S phase of the cell cycle. Inhibition of Cdk2 activity in vitro has been shown to protect cultured sympathetic neurons and heart muscle cells from apoptosis (26, 27). Our results in noncycling CD4+CD8+ thymocytes provide the first evidence that Cdk2 has a crucial role in the induction of cell death during normal development. However, it has been shown that Cdk2 inhibition can also lead to cell death in tumor cell lines, and Cdk2 is frequently deregulated in tumors (28). Similarly, we found that in contrast to developing, noncycling thymocytes, inhibition of Cdk2 in four different thymic lymphoma cell lines led to rapid apoptosis and sensitized T cell tumors to anti-CD3, dexamethasone, or γ-irradiation-mediated cell death (not shown). These results suggest that Cdk2 has functions in the apoptotic processes that regulate normal development which are distinct from those in tumorigenesis and transformation. Thus, specific inhibition of Cdk2 could be exploited to sensitize tumor cells to apoptosis by anticancer drugs, whereas molecular inhibition of Cdk2 might protect normal, noncycling cells from the adverse effects of the same drugs.

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