A Role for G1/S Cyclin-dependent Protein Kinases in the Apoptotic Response to Ionizing Radiation*

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In Xenopus development the mid-blastula transition (MBT) marks a dramatic change in response of the embryo to ionizing radiation. Whereas inhibition of cyclin D1-Cdk4 and cyclin A2-Cdk2 by p27Xic1 has been linked to cell cycle arrest and prevention of apoptosis in embryos irradiated post-MBT, distinct roles for these complexes during apoptosis are evident in embryos irradiated pre-MBT. Cyclin A2 is cleaved by caspases to generate a truncated complex termed ΔN-cyclin A2-Cdk2, which is kinase active, not inhibited by p27Xic1, and not sensitive to degradation by the ubiquitin-mediated proteasome pathway. Moreover, ΔN-cyclin A2-Cdk2 has an expanded substrate specificity and can phosphorylate histone H2B at Ser-32, which may facilitate DNA cleavage. Consistent with a role for cyclin A2 in apoptosis, the addition of ΔN-cyclin A2-Cdk2, but not full-length cyclin A2-Cdk2, to Xenopus egg extracts triggers apoptotic DNA fragmentation even when caspases are not activated. Similarly, cyclin D1 is targeted by caspases, and the generated product exhibits higher affinity for p27Xic1, leading to reduced phosphorylation of the retinoblastoma protein (pRB) during apoptosis. These data suggest that caspase cleavage of both cyclin D1-Cdk4 and cyclin A2-Cdk2 promotes specific apoptotic events in embryos undergoing apoptosis in response to ionizing radiation.

Exposure of mammalian cells to damaging agents can result in transient cell cycle arrest or in apoptotic cell death. Although the processes of cell death and cell proliferation appear to be opposing and mutually contradictory, substantial evidence now indicates that the two processes are linked (1–3). Transitions between different cell cycle phases are regulated by surveillance mechanisms or checkpoints that monitor the integrity of the DNA. Cyclin-dependent kinase (Cdk) complexes essential for cell cycle transitions are controlled by checkpoints, and inappropriate Cdk activity during cell cycle transitions often correlates with apoptosis. For example, in some systems induction of apoptosis by various stimuli requires the activation of either Cdc2 or Cdk2 (4–8), whereas forced expression of Cdk inhibitors prevents apoptosis in various cell types (8–11). Consistent with a role for cyclins in apoptosis, cyclin E overexpression greatly sensitizes cells to radiation, whereas its inhibition by a dominant-negative Cdk2 blocks cell death (9, 12). In addition, neuronal apoptosis is accompanied by changes in Cdk activity and cyclin D expression (13), and expression of the CKIs p16, p21, and p27 or dominant-negative forms of Cdk4 and -6 inhibits death in sympathetic neurons caused by NGF withdrawal (14).

D-type cyclins, which are activated by rearrangement or amplification in several tumors, interact with two distinct catalytic partners, Cdk4 and Cdk6, to yield different holoenzymes that are expressed in tissue-specific patterns (for review, see Ref. 15). These complexes phosphorylate the retinoblastoma protein (pRB, a tumor suppressor gene product) and regulate the G1/S transition in the cell cycle (16). In Xenopus, cyclin D1 expression, both at the mRNA and protein levels, starts at the time of mid-blastula transition (MBT)1 during early development, although Cdk4 is already present in oocytes. Cyclin A is a key regulatory protein involved in both S phase and the G2/M transition of the cell cycle through its association with Cdk2 and Cdc2, respectively. Two types of cyclin A, A1 and A2, have been described in Xenopus (17). Cyclin A1 is present in unfertilized eggs and in early cleavage stages and disappears rapidly after the MBT. Cyclin A2 protein is very low in early embryos, increases rapidly at the MBT, and reaches a constant level in adult tissues (17). It shows a greater similarity to human cyclin A than to Xenopus cyclin A1.

Interestingly, in Xenopus embryos cyclin A1 activity has been implicated in a dramatic change in the response to DNA damage at the MBT. When ionizing radiation is administered any time before the MBT, Xenopus embryos initiate apoptosis after the MBT and exhibit prolonged activation of cyclin A1-Cdk2 (18–20). However, if ionizing radiation is given after the MBT, apoptosis is prevented by multiple mechanisms, including the inactivation of proapoptotic components, activation of antiapoptotic elements, and arrest of cell cycle progression in G1 (21). The latter is a direct consequence of an increased amount of the Cdk inhibitor p27Xic1, which binds to and inhibits both cyclin D1-Cdk4 and cyclin A2-Cdk2 complexes. This promotes a delay in the G1/S transition, allowing more time for DNA repair, and blocks apoptosis, which might occur if S phase were initiated with damaged DNA (21).

Nearly all programmed cell death is executed by a family of aspartate-directed cysteine proteases known as caspases (for review, see Ref. 22). Many proteins targeted by caspases are involved in RNA splicing, DNA repair, and scaffolding of pro-

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1 The abbreviations used are: MBT, mid-blastula transition; Cdk, cyclin-dependent kinase; CKI, Cdk inhibitor; CAD, caspase-activated DNase; CSF, cytostatic factor; wt, wild type; CHO, Chinese hamster ovary; GST, glutathione S-transferase; MPF, maturation-promoting factor.
Role of Cdks in Apoptosis

38477

teins in the cytosol and nucleus, although in most cases their exact roles in execution of the apoptotic program remain unclear. Emerging evidence has also identified protein kinases as caspase targets. Some of these kinases are activated indirectly through caspase action on other substrates, but an increasing number have been found to be directly cleaved by caspases, leading to modulation of their catalytic activity (for review, see Ref. 23). Whereas the caspase-cleaved forms of PAK2, MAPK kinase kinase, focal adhesion kinase, protein kinase Cθ, and calmodulin kinase II are active, cleavage of DNA-protein kinase, Raf-1 and Akt during apoptosis correlates with loss of activity. Other studies show that histones become phosphorylated in response to apoptosis-inducing signals (24, 25). The timing of phosphorylation of histone H2B on Ser-32 coincides with the initiation of DNA fragmentation seen at early stages of apoptosis. Some evidence supports a role for protein kinase Cθ in histone H2B phosphorylation inasmuch as it is cleaved by caspases and phosphorylates histone H2B at Ser-32 (26).

Here we show that apoptosis in Xenopus is associated with cleaved forms of cyclin A2-Cdk2 and cyclin D1-Cdk4 generated by caspase activation. Both cleaved forms exhibit alterations in their kinase activity and in regulation by inhibitory proteins. In addition, the cleaved form of cyclin A2-Cdk2 contributes to nuclear apoptosis, and the cleaved form of cyclin D1-Cdk4 binds p27Kip1 with higher affinity, leading to reduced phosphorylation of pRB during apoptosis.

Experimental Procedures

Preparation of Embryos—Eggs were fertilized in vitro as described previously (27), and embryos were staged according to Nieuwkoop and Faber (28). For time-course experiments, embryos were irradiated at either stage 6 or 9, collected as the indicated two-day-embryos, frozen on dry ice, and stored at 80°C. Embryos were homogenized and processed for pull-down analysis as described (18, 29). γ-Irradiation was performed by exposing embryos to 20 gray (2000 rads) from a 60Co source as described (18).

Preparation of Xenopus Egg Extracts—Metaphase II-arrested cytosolic (CSF) extracts were prepared from Xenopus eggs as described (30). Extracts were supplemented at time 0 with 500 μM CaCl2, 50 μg/ml cycloheximide, and demembranated sperm nuclei (1000 nuclei/μl) and incubated at room temperature for 1 h, at which time the extracts were in interphase. Recombinant proteins or buffer was then added to these extracts, and 2-μl aliquots were removed at regular intervals and analyzed for apoptosis by fluorescence microscopy after formaldehyde fixation (3.7% formaldehyde, 48% glycerol) and staining with 1 μg/ml Hoechst 33342 (1.5 μM MMR (100 mM NaCl, 2 mM KCl, 1 mM MgSO4, 2 mM CaCl2, 5 mM HEPES, pH 7.8). Assay of Apoptosis in a Cell-free System—The assay was performed according to conditions described previously (20) with the following modifications. Embryos were irradiated at either stage 6 or stage 9 and collected at different times after irradiation. In substrate cleavage assays, 35S-labeled Xenopus cyclin D1 or cyclin A2 translated in vitro (TNT-coupled reticulocyte lysate system, Promega) was added at a 1:10 dilution into an extract volume equivalent to one embryo. Samples were incubated at 30°C, and aliquots of 3 μl were withdrawn at various times and diluted with 6× SDS-PAGE sample buffer. The cleavage products were resolved by SDS-PAGE and visualized by autoradiography. Caspase inhibitors (N-acetyl-DEVD-aldehyde (DEVD)), N-acetyl-LEHD-aldehyde (LEHD), N-acetyl-IETD-aldehyde (IETD), N-acetyl-YVAD-aldehyde (YVAD), N-acetyl-VEID-aldehyde (VEID); BIOMOL Research Laboratories) were added to apoptotic extracts (250 nm final concentration) and incubated for 20 min at 30°C before the addition of the radiolabeled Xenopus cyclin.

Production, Purification, and Assay of Recombinant Cyclin A2-Cdk2, ΔN-cyclin A2-Cdk2, Cyclin D1-Cdk4, and ΔC-cyclin D1-Cdk4 Complexes—Cyclin A2-Cdk2, cyclin D1-Cdk4, and p27Kip1 were expressed in baculovirus-infected Sf9 cells as fusion proteins with glutathione S-transferase, and the kinase dead form of Cdk2 was generated by site-directed mutagenesis to change Asp-145 to Asn. Their kinase activity and in regulation by inhibitory proteins. In addition, the cleaved form of cyclin A2-Cdk2 contributes to nuclear apoptosis, and the cleaved form of cyclin D1-Cdk4 binds p27Kip1 with higher affinity, leading to reduced phosphorylation of pRB during apoptosis.

RESULTS

Our previous results showed that G1 arrest of the cell cycle occurs during prevention of apoptosis in embryos irradiated after the MBT. The G1 arrest is a direct consequence of an increased level of p27Kip1, which binds to and inhibits both Cdk2 and Cdk4 complexes (21). Embryos irradiated before the MBT undergo apoptosis beginning several hours after the MBT. We studied whether cyclin-Cdk complexes are implicated in the induction or execution of apoptosis in Xenopus embryos irradiated before the MBT. Initially, cyclin A2-Cdk2 or cyclin D1-Cdk4 complexes were examined after irradiation in vitro because cyclin complexes A1 and E are largely degraded after the MBT, and cyclin B-Cdk2 complexes are not active during apoptosis (18, 35). In addition, Stack and Newport (20) report that cyclin A2 is cleaved by caspases in response to prolonged activation of the DNA replication checkpoint by hydroxyurea. However, its possible role in apoptosis has not been examined.
Embryos were irradiated at either stage 6 (pre-MBT) or stage 9 (post-MBT) and collected at different times. Extracts were incubated with p13 Suc1 beads, which specifically bind Cdk2 and Cdc2 complexes, and the bound complexes were analyzed by Western blotting with anti-cyclin A2 antibody. Lower panel, embryo lysates were resolved by SDS-PAGE and blotted with anti-cyclin D1 antibody. The arrows on the right denote cyclin A2 and D1 and the cleavage fragments. EGT, early gastrula transition. Molecular mass markers (in kDa) are indicated on the left. B, embryo extracts were incubated with radiolabeled cyclin D1 as described under “Experimental Procedures.” At the indicated times, aliquots were removed and analyzed for cyclin D1 cleavage by SDS-PAGE and autoradiography. C, at the indicated times, samples equivalent to three embryos were tested for the activity of caspase-2, -3, and -7 using a specific colorimetric substrate as described under “Experimental Procedures.” Samples were assayed in triplicate, and the data are presented as the mean ± S.E.

Embryos were irradiated at either stage 6 (pre-MBT) or stage 9 (post-MBT) and collected at different times. Extracts were incubated with p13 Suc1 beads, which specifically bind Cdk2 and Cdc2 complexes, and the bound complexes were analyzed by Western blotting with an anti-cyclin A2 antibody. Cyclin A2 was cleaved in embryos irradiated before the MBT (Fig. 1A, upper panel), whereas cleavage of cyclin A2 did not occur in embryos irradiated post-MBT (data not shown). No cleavage of cyclin B was evident in embryos irradiated before or after the MBT (data not shown). The cleaved form of cyclin A2 was present as a doublet, probably the result of phosphorylation, as it migrated as one band when p13 Suc1 precipitates were incubated with acid phosphatase (data not shown). In addition, the formation of the cleaved product correlates in time with the appearance of the apoptotic phenotype during the onset of gastrulation in irradiated embryos, including chromatin condensation, DNA fragmentation, and membrane blebbing (18).

The presence of the cyclin A2 fragment on p13 Suc1 bead precipitates indicates that the cleaved form of cyclin A2 is still able to bind its Cdk2 partner. Because our previous work suggested that inhibition of both cyclin A2-Cdk2 and cyclin D1-Cdk4 by p27Nip1 contributes to G1 arrest and prevention of apoptosis in post-MBT-irradiated embryos (21), we next investigated whether cyclin D1 might also be cleaved in apoptotic embryos. Immunoprecipitation and Western blot analysis of cyclin D1 in embryos irradiated before the MBT revealed the appearance of a cleavage product at the onset of gastrulation (Fig. 1A, lower panel). As was the case with cyclin A2 (20, 21), the cleavage could also be observed if cell-free lysates of embryos undergoing apoptosis were incubated with [35S]methionine-labeled cyclin D1 (Fig. 1B).
Further studies were devoted to determining which proteases are responsible for cleavage of each cyclin. Initial attention was focused on caspases, a family of cysteine-dependent proteases that cleave substrates C-terminal to a conserved aspartate residue (22). Caspase assays with specific colorimetric substrates (36, 37) revealed that caspase-2, -3, and -7 activity was increased after the MBT (Fig. 1C) at the time when cyclin D1 and A2 cleavage occurs (Fig. 1A). To investigate directly whether caspases are responsible for cyclin cleavage, we monitored the degradation of radiolabeled cyclins added to apoptotic extracts in the presence of specific caspase inhibitors. Inhibitor selectivities are based on the high specificity of each caspase for a cleavage site flanked by residues N-terminal to aspartate (37). Treatment of embryo extracts with the inhibitor DEVD prevented cyclin A2 and D1 cleavage, whereas no effect was observed with any other inhibitor tested (Fig. 2, A and B). These results suggest that the cleavage of cyclins A2 and D1, which occurs after irradiation, might be due to members of the caspase-3 subfamily. One prototypical consensus site of cleavage for caspase-3 is situated in the C-terminal region of Xenopus cyclin D1 at position 275–278 (DEVD) (37). The truncated form of cyclin D1, termed ΔC-cyclin D1, generated in vivo after caspase activation (Fig. 1A), has a molecular weight identical to that observed after in vitro caspase cleavage (Fig. 2D). Interestingly, whereas the cleavage site of cyclin A2 has an Asp residue in the P4 position, the overall sequence (87DEPD90) does not represent a prototypical caspase-3 consensus sequence (37). This motif was changed to AEKD (A2D87A), DEPA (A2D90A), and AEPA (A2D87A/D90A), respectively, by site-directed mutagenesis, and the mutant proteins were analyzed for cleavage in the cell-free assay. Wild-type cyclin A2 (A2) was cleaved, whereas cleavage of A2D87A, A2D90A, and A2D87A/D90A was not detected (Fig. 2C). Asp-90 is the same cleavage site identified in cyclin A2 when embryos undergo apoptosis after prolonged hydroxyurea treatment (20). Purified cyclin-Cdk complexes produced in SF9 cells were then used to examine whether they are direct substrates for caspases. These fusion proteins were incubated with various caspases in vitro, separated by SDS-PAGE, and analyzed by immunoblotting with anti-cyclin A2 and D1 antibodies. Fig. 2D, upper panel, shows that cyclin A2 is efficiently cleaved by caspases-2, -3, and -7 but not by caspases-6, -8, and -9, and cyclin D1 cleavage was evident only with caspases-3 and -7 (Fig. 2D, lower panel). The appearance of the fragments could be effectively prevented by preincubating the purified proteins with DEVD-CHO before the in vitro cleavage assay (data not shown).

We previously reported that the Xenopus Cdk inhibitor p27Xic1 binds to and inhibits cyclin A2-Cdk2 beginning shortly after irradiation post-MBT, when no caspase activity is detected (21). However, eventually caspase activation and apoptosis occur in such embryos if DNA repair is not complete. Because p27 Xic1 is not a caspase substrate, 2 it does not protect the complex from caspase-mediated cleavage.

Cleavage of cyclin A2 at Asp-90 also removes the destruction of p27 Xic1, as demonstrated by Western blotting and autoradiography. Fig. 2E shows that addition of radiolabeled cyclin A2, cyclin A2-Cdk2, and cyclin A2-Cdk2-p27Xic1 complexes to extracts from non-irradiated embryos (Control) inhibited cyclin A2 cleavage. After irradiation (γ-IR), cyclin A2 cleavage was increased, and the addition of active recombinant caspase-3 partially inhibited cleavage. However, the addition of inactive caspase-3 (DEVD-CHO) did not inhibit cleavage. Purified cyclin A2-Cdk2-p27Xic1 complexes were incubated with either control γ-IR extracts, irradiated at stage 6 (γ-IR) and collected 6 h after the MBT were incubated with 250 nM of the indicated caspase inhibitor before the addition of radiolabeled cyclin D1. Ac-DEVD-CHO, Ac-IETD-CHO, Ac-YVAD-CHO are aldehyde inhibitors of the subfamily of caspase-3, -6 and -8, -9, and caspase-1 respectively. Where indicated, MeSO (DMSO) was added as a vehicle control. B, extracts prepared as described in panel A were incubated with the indicated caspase inhibitors and evaluated for effects on cleavage of radiolabeled cyclin D1. C, radiolabeled mutant forms of cyclin A2 (D87A, D90A, D87A/D90A) were added to either control or γ-IR extracts, incubated at 30 °C for the indicated times, and analyzed for cleavage by SDS-PAGE and autoradiography. D, purified cyclin A2-Cdk2 (upper panel) or cyclin D1-Cdk4 (lower panel) were incubated with recombinant active caspases-2, -3, -6, -7, -8, -9, and -10 (lacking the pro-domain; 15 units/μl) for 30 min at 30 °C. Samples were separated by SDS-PAGE and analyzed by Western blotting with anti-cyclin A2 or D1 antibodies. E, purified cyclin A2-Cdk2 and cyclin A2-Cdk2-p27Xic1 (upper panel) or cyclin D1-Cdk4 and cyclin D1-Cdk4-p27Xic1 complexes were incubated with either recombinant caspase-3 (15 units/μl) or buffer at 30 °C as indicated. Samples were removed at the indicated times and analyzed by Western blotting with an anti-cyclin A2 or D1 antibody.

2 C. V. Finkielstein, L. G. Chen, and J. L. Maller, unpublished data.
box required for ubiquitin-mediated degradation and, thus, might ensure the persistence of the cleaved complex. To examine this possibility directly, we used a cell-free system based on extracts from *Xenopus* unfertilized eggs arrested at metaphase of meiosis II by a calcium-sensitive activity named cytostatic factor (CSF), which stabilizes MPF activity. The addition of calcium triggers CSF release, resulting in cyclin A and B degradation, a decline in MPF activity, and entry into interphase of the first mitotic cycle. When cycloheximide was also added to these extracts, *de novo* synthesis of cyclins was prevented, and the extracts remained in interphase (interphase extracts). Purified cyclin A2-Cdk2 or ΔN-cyclin A2-Cdk2 were added to a CSF-arrested extract, and the level of various cyclin components was assessed by immunoblotting at different times after calcium addition. Release from CSF was confirmed by degradation of endogenous cyclin B1 (Fig. 3A, *upper panel*). The level of ectopic cyclin A2 also dramatically decreased 15 min after calcium addition, and no detectable cyclin A2 was present at later times (Fig. 3A, *middle panel*). In contrast, the level of ectopic ΔN-cyclin A2 remained constant throughout the time course analyzed (Fig. 3A, *lower panel*), supporting the requirement of a destruction box for cyclin A2 degradation and its removal by caspase cleavage during apoptosis.

We explored further the functional relationship between cyclin cleavage and apoptosis by analyzing the activity of the endogenous cyclin A2-Cdk2 complex in embryos irradiated before the MBT. Extracts were immunoprecipitated with cyclin A2 antibody, and cyclin A2-Cdk2 activity was measured using histone H1 as substrate. Fig. 3B reveals that cyclin A2 cleavage after irradiation *in vivo* results in no loss of cyclin A2-Cdk2 histone H1 kinase activity (*upper panel*). Immunoprecipitation of equal amounts of cyclin A2-Cdk2 and ΔN-cyclin A2-Cdk2 was confirmed by Western blot analysis of the Cdk2 protein in each sample (Fig. 3B, *lower panel*). However, an important difference was found between the full-length and cleaved form of cyclin A2-Cdk2; assays *in vitro* with complexes purified from baculovirus-infected Sf9 cells showed that the ΔN-cyclin A2-Cdk2 complex is no longer subject to inhibition by p27∗Xic1 (Fig. 3C). These results suggest that p27∗Xic1 is unable to bind ΔN-cyclin A2-Cdk2. To assess this possibility, the presence of p27∗Xic1 in the complex was examined using p13Suc1 beads to precipitate cyclin A2-Cdk2 after cleavage *in vitro* by caspase 3. Fig. 3D shows that p27∗Xic1 is released from the complex after caspase cleavage of cyclin A2 *in vitro*. In light of these *in vitro* results, it was important to evaluate whether cyclin A2-Cdk2 from irradiated embryos undergoing DNA damage *in vivo* is also insensitive to inhibition by p27∗Xic1. As shown in Fig. 3E, cyclin A2-Cdk2 immunoprecipitates from embryos irradiated *in vivo* show little inhibition by 375 nM p27∗Xic1, whereas controls are almost completely inhibited at this concentration. Thus, ΔN-cyclin A2-Cdk2 activity is not subject to regulation by either ubiquitin-mediated degradation or by binding of p27∗Xic1.

Remarkably, similar experiments with ΔC-cyclin D1-Cdk4 revealed that *in vitro* the cleaved complex is inhibited 10-fold more potently by p27∗Xic1 than is the case with the uncleaved form of the enzyme (Fig. 4A). We next tested whether increased inhibition is also evident *in vivo* after irradiation. Initially, the cyclin D1-Cdk4 complex was immunoprecipitated from embryos irradiated pre-MBT, and its kinase activity was determined using pRB as substrate. The reduced activity detected after irradiation (Fig. 4B, *upper panel*) correlated with the presence of the cleaved form of cyclin D (Fig. 1A, *lower panel*) and did not result from an increased amount of endogenous p27∗Xic1 (Fig. 4B, *lower panel*). Inhibition of cyclin D1-Cdk4 activity *in vivo* could also be observed as loss of an electrophoretically shifted form of endogenous pRB (Fig. 4B, *middle panel*). To assess directly whether cyclin D1-Cdk4 cleavage *in vivo* increases p27∗Xic1 binding, myc-tagged p27∗Xic1 was injected into one-cell stage embryos, and binding to either cyclin A2-
Cdk2 or cyclin D1-Cdk4 was assessed by immunoblotting the myc epitope on p13Suc1 beads or Cdk4 immunoprecipitates, respectively. The results demonstrate that more p27Xic1 is bound to cyclin D1-Cdk4 after irradiation, whereas a reduced amount of the inhibitor is detected after precipitation with p13Suc1 beads, perhaps reflecting the reduced ability of p27Xic1 to bind ΔN-cyclin A2-Cdk2 (Fig. 4C).

These results suggest that during radiation-induced apoptosis, the activity of cyclin A2-Cdk2 is sustained, whereas cyclin D1-Cdk4 is down-regulated. To determine the physiological significance of the ΔN-cyclin A2-Cdk2 complex in the regulation of cell death, we examined the effect of the recombinant complex in interphase extracts, which have been widely used to study apoptosis induced by various agents (39–41). In egg extracts containing mitochondria, the apoptotic process is dependent on the release of cytochrome c (42, 43), and apoptotic nuclear morphology, including chromatin condensation and nuclear fragmentation, normally appears within 2–4 h (42, 44). We examined the consequence of the addition of the ΔN-cyclin A2-Cdk2 complex to interphase extracts. As visualized by fluorescence microscopy, nuclei underwent rapid degeneration with formation of pyknotic DNA bodies between 80 and 100 min after ΔN-cyclin A2-Cdk2 wt addition in a manner morphologically identical to that obtained by incubation of the extract with cytochrome c (Fig. 5A, upper panel). To assess whether DNA fragmentation occurred, fluorescence-activated cell sorting was performed on nuclei isolated from apoptotic embryos. In this assay, DNA fragmentation is revealed by formation of pools of DNA smaller than the diploid G1 level (sub-G1 peak). Nuclear fragmentation induced in response to ΔN-cyclin A2-Cdk2 wt addition produced a characteristic sub-G1 peak indicative of cleavage of the DNA (Fig. 5A, lower panel). In contrast, no apoptotic features were evident after the addition of the same amount of wild type cyclin A2-Cdk2 activity or kinase-dead ΔN-cyclin A2-Cdk2 (Fig. 5A, lower panel). In addition, preincubation of the ΔN-cyclin A2-Cdk2 wt complex with p27Xic1 did not abolish the apoptotic activity manifested in an interphase extract (Fig. 5A, upper panel), as expected, since p27Xic1 is unable to inhibit ΔN-cyclin A2-Cdk2 activity (Fig. 5C). One possibility is that apoptosis mediated by ΔN-cyclin A2-Cdk2 merely reflects a feedback loop that leads to caspase activation. To test this possibility, either cytochrome c or ΔN-cyclin A2-Cdk2 wt were added to interphase extracts, and samples were removed and labeled with a biotinylated affinity-labeling reagent, zEK(bio)D-aomk, which can detect as little as 1 ng of a purified caspase (32). This reagent mimics the peptide sequences preferred by caspases and binds irreversibly to the active site cysteine within the large subunit of most active caspases; in some systems it can detect even background levels of active caspases that are not sufficient to induce apoptosis (32, 37). Fifteen minutes after the addition of cytochrome c to an interphase extract, six discrete zEK(bio)D-aomk-reactive bands were detected in CSF extracts (Fig. 5B, upper panel). Previous reports (45) and comparison with affinity-labeled recombinant caspases suggests that at least 2 of the bands correspond to active forms of caspase-3 and -6 (data not shown). Cleavage of radiolabeled cyclin A2 added to an interphase extract also supports caspase-3 activation after cytochrome c addition (Fig. 5B, lower panel). However, both affinity labeling and experiments with radiolabeled cyclin A2 indicate that ΔN-cyclin A2-Cdk2 expression does not promote caspase activation (Fig. 5B, upper panel). This suggests that ΔN-cyclin A2-Cdk2 is sufficient to directly control the changes in morphology and cleavage of DNA that occur during apoptosis in egg extracts.

An important question concerns what substrate for cyclin A2-Cdk2 might be involved in promoting DNA cleavage. Studies in other laboratories implicate histone phosphorylation in promoting cleavage, especially that of histone H2B (24). Although histone H1 is the best-characterized in vitro sub-
strate for Cdk2 (46), we investigated whether ΔN-cyclin A2-Cdk2 might have an altered activity toward phosphorylation of other histones. Remarkably, whereas both complexes were able to phosphorylate histone H1 with similar specific activity, only ΔN-cyclin A2-Cdk2 was able to phosphorylate histone H2B (Fig. 6A). The activity of ΔN-cyclin A2-Cdk2 toward both histone H1 and H2B was inhibited by olomoucine (data not shown), a specific inhibitor of Cdkks (47). Little change in the phosphorylation of histones H3 and H4 was evident with ΔN-cyclin A2-Cdk2. Phosphorylation of histone H2B by ΔN-cyclin A2-Cdk2 occurred predominantly on serine residues, although some phosphothreonine was also detectable (Fig. 6B). Several reports have shown that during apoptosis in mammalian cells histone H2B is rapidly phosphorylated, and this phosphorylation event correlates tightly with nucleosome cleavage of the DNA. This reaction has been proposed to be mediated by protein kinase C, based on the sequence around Ser-32 (24). To examine whether ΔN-cyclin A2-Cdk2 might phosphorylate this site, we carried out in vitro assays with a synthetic peptide encompassing Ser-32 (KKRKRS32RK). As shown in Fig. 6C, the H2B peptide was phosphorylated significantly by ΔN-cyclin A2-Cdk2 but not by full-length cyclin A2-Cdk2. To address whether ΔN-cyclin A2-Cdk2 generated in vivo during apoptosis has kinase activity against histone H2B, Xenopus embryos were irradiated before the MBT, and the ΔN-cyclin A2-Cdk2 complex was precipitated with p13Suc1 beads. H2B kinase activity was detected only in apoptotic embryos and was correlated with the presence of the cleaved form of cyclin A2 (Fig. 6D). Then we asked whether increased phosphorylation of histone H2B at Ser-32 occurs during apoptosis in Xenopus egg extracts. Histone H2B was added to interphase extracts in the presence of inhibitors of several protein kinases known to phosphorylate H2B, then ΔN-cyclin A2-Cdk2 was generated in situ by the addition of either cytochrome c or caspase-3 and cyclin A2-Cdk2. Samples were taken at different times and analyzed for phosphorylation of histone H2B at Ser-32 using a phosphospecific antibody. Ser-32 phosphorylation occurred only when ΔN-cyclin A2-Cdk2 was present (Fig. 6E). Moreover, all activity against Ser-32 in H2B could be depleted by p13Suc1 beads (data not shown), suggesting ΔN-cyclin A2-Cdk2 is the enzyme responsible for Ser-32 phosphorylation in the extract.
systems suggest that cyclins D and A take part in programmed cell death, but no specific role for these cyclins has been identified (20, 49, 50). Interestingly, our data support a novel role for cyclin A2 in mediating a specific event in apoptosis, DNA fragmentation. One of the most remarkable features of ΔN-cyclin A2-Cdk2 is its expanded substrate specificity to include non-proline-directed sites. To our knowledge this is the first example of such a profound change in protein kinase substrate specificity as a result of protease cleavage. Inasmuch as no change in cyclin A2-Cdk2 activity toward histone H1 was detected in apoptotic embryos, the irreversible proteolytic cleavage of cyclin A2 by caspases may act as a switch to expand cyclin A2-Cdk2 substrate specificity to irreversibly promote apoptosis. In addition, we also found that cyclin D1-Cdk4 became a caspase substrate. Its binding capacity for p27Xic1 was increased, and this resulted in a reduced capacity to phosphorylate pRB in vitro and in vivo.

The destruction of mitotic cyclins by ubiquitination and inhibition of cyclin-Cdk complexes by Cdk inhibitors (CKIs) are important elements in cell cycle control. Here we have shown that caspase-dependent proteolytic cleavage is an additional mechanism used by Xenopus cells to regulate the functions of cyclins D1 and A2 during apoptosis. The apoptosis dependence, sensitivity to caspase inhibitors, and in vitro cleavage of cyclins D1 and A2 indicate that ΔN-cyclin A2 and ΔN-cyclin D1 are both generated during apoptosis after proteolytic attack by a caspase. Our data clearly indicate that caspase-3 or a caspase-3-like enzyme directly cleaves both cyclin A2 and D1 since (i) generation of ΔN-cyclin A2 and ΔN-cyclin D1 is prevented when cell-free extracts are treated with the specific caspase-3 (DEVD) inhibitors but not when inhibitors of caspase 1, 6, or 8 are used (Fig. 2A), (ii) ΔN-cyclin A2 and ΔN-cyclin D1 can be generated in vitro in a reaction containing either cyclin A2 or D1 and purified caspase 3 (Fig. 2, B and C), and (iii) cyclin A2 cleavage is prevented when Aap-87 and Aap-90 are mutated (data not shown and Ref. 20). Interestingly, cyclin A2 but not D1 seems to be cleaved by caspase-2. Caspase-2, -3, and -7 each display similar specificities (37, 51). They share a strong requirement for Asp at the P4 position, although caspase-2 also requires a P5 hydrophobic residue for efficient cleavage, a structural context that is present in cyclin A2.

The cell cycle-dependent proteolysis of mitotic A- and B-type cyclins relies on a conserved motif of nine residues, the destruction box, which is located 40–50 residues from the N terminus (52). Inhibition of cyclin A/Cdk activity by CKIs requires simul-
taneous interaction of CKIs with both the Cdk2 ATP-binding site and the N-terminal 120 amino acids of cyclin A (38, 53). During apoptosis, the caspase-mediated cleavage of Xenopus cyclin A2 at the $^{87}$DEPD$^{90}$ site removes both the putative destruction box and the CKI interaction motif, leading to the formation of active $\Delta$N-cyclin A2-Cdk2, which is insensitive to both degradation and inhibition by CKIs (Figs. 2 and 3). The irreversibility of the cyclin A2-Cdk2 activation after caspase cleavage supports the idea that $\Delta$N-cyclin A2-Cdk2 is a mediator of apoptotic processes.

Phosphorylation of the retinoblastoma protein (pRB) is catalyzed by cyclin-Cdk during G$_1$ progression and inactivates the growth-suppression function of pRB (16). Cleavage of pRB has been observed in a number of cell types upon exposure to genotoxic drugs, CD95 (Fas), or tumor necrosis factor (for review, see Refs. 54 and 55), and the degradation could be prevented by treating cells with a caspase inhibitor (55). A caspase consensus cleavage site, DEADG, is found in the human pRB sequence at amino acids 883–887, and this site is conserved in mouse, chicken, and Xenopus pRB (56, 57). Cleavage generates $\Delta$pRB, which is shortened by 42 amino acids, and the roughly 5-kDa smaller cleaved product is more sensitive to degradation by other proteases. It appears that in apoptotic Xenopus embryos pRB is not only dephosphorylated but also subsequently cleaved to generate a product similar in molecular weight to human $\Delta$pRB (Fig. 4B, middle panel, open arrow).

It has been proposed that execution caspases exert their roles either by blocking pathways that might interfere with the apoptotic program or by activating pathways that advance the program or both (58). By treating interphase extracts with $\Delta$N-cyclin A2-Cdk2, we were able to evaluate its role in triggering the nuclear events associated with apoptosis in the absence of other caspase-dependent apoptotic initiators. Chromatin was condensed into discrete domains, and the nuclei were eventually fragmented and destroyed even in the absence of detectable caspase activation (Fig. 5). The observed DNA fragmentation was probably the result of the action of preexisting nuclear DNAases. At least two parallel and redundant pathways are known that can lead to nuclear apoptosis. One of these pathways involves a caspase-activated DNAase (variously named CAD, CPAN, or DFF40) and leads to nucleosomal DNA fragmentation and advanced chromatin condensation (for review, see Ref. 59). The second, caspase-independent pathway involves molecules such as apoptosis-inducing factor and leads to nucleosomal and large scale DNA fragmentation through the activation of endogenous endonucleases and peripheral chromatin condensation (60). Moreover, both pathways can act in a redundant fashion, as suggested by studies in which nuclear apoptosis is prevented only when both CAD and apoptosis-inducing factor are inhibited (60). Although $\Delta$N-cyclin A2-Cdk2 can promote nuclear apoptosis through the action of endogenous nucleases in interphase extracts (Fig. 5), it is likely that both caspase-dependent and -independent mechanisms act in vivo in embryos, since a caspase-dependent nuclease like DFF40 might be activated at the same time that $\Delta$N-cyclin A2-Cdk2 is formed. It is evident that the study of non-CAD-dependent DNA cleavage might prove useful using egg extracts supplemented with $\Delta$N-cyclin A2-Cdk2.

Several studies have shown that phosphorylation of mammalian histones is triggered by apoptosis-induced signals. Phosphorylation of both histones H2A.X and H2B is dependent on activation of caspases and, therefore, may be linked to caspase-induced signaling pathways (24, 61). The H2B phosphorylation site is located in the inner globular region of the N-terminal tail at Ser-32 (24) and is associated with the early phase of DNA fragmentation and linked to caspase-induced signaling pathways (24, 62). Ser-32 can be phosphorylated in vitro by protein kinase C$\delta$ and to a lesser extent by protein kinase A (24), as predicted by the amino acid sequence around the phosphorylation site. Our data suggest the potential importance of the $\Delta$N-cyclin A2-Cdk2 complex in induction of apoptosis through the phosphorylation of histone H2B at Ser-32. The concept that apoptosis involves active Cdks has been evident for some time from studies in many laboratories (4, 5, 18, 49). However, two aspects of Cdk activation reported here are novel. First, the cleaved form of cyclin A2-Cdk2 has expanded substrate specificity to include a protein kinase C-like consensus sequence that does not have a proline C-terminal to the phosphorylation site. Remarkably, this occurs without any change in the specific activity toward histone H1 (Fig. 6). Whether altered substrate specificity will prove to be a general property of kinases that are active after caspase cleavage is an intriguing possibility. Second, our results show that in the egg extract nuclear apoptosis can be elicited by $\Delta$N-cyclin A2-Cdk2 even in the absence of detectable caspase activation, establishing this Cdk as sufficient to promote DNA fragmentation. Whether this prominent role for cyclin A2-Cdk2 is conserved in other apoptotic systems remains to be established. However, in Xenopus development it is evident that cyclin A2-Cdk2 mediates not only cell cycle arrest when apoptosis is blocked (21) but also promotes DNA fragmentation when the cell death program is activated (Fig. 7).

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