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A Role for G_1/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 $p27^{Xic1}$ 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 $p27^{Xic1}$, 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 $p27^{Xic1}$, 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 G_1/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 G_2/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 G_1 (21). The latter is a direct consequence of an increased amount of the Cdk inhibitor $p27^{Xic1}$, which binds to and inhibits both cyclin D1-Cdk4 and cyclin A2-Cdk2 complexes. This promotes a delay in the G_1/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.

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 Cδ, 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 Cδ 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 $p27^{Xic1}$ 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 stage 9, collected at the indicated times, 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 ^{60}Co source as described (18).

Preparation of Xenopus Egg Extracts—Metaphase II-arrested cytostatic factor (CSF) extracts were prepared from *Xenopus* eggs as described (30). Extracts were supplemented at time 0 with 500 μM $CaCl_2$, 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 4,6-diamidino-2-phenylindole in 1× MMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 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, ^{35}S-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—*Xenopus* Cdk2 and $p27^{Xic1}$ 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. *Xenopus* cyclin A2 and ΔN-cyclin A2 were cloned into pVL1392 and pBac-2cp (Novagen), respectively. Sf9 cells were co-infected with baculoviruses expressing either *Xenopus* cyclin A2 or ΔN-cyclin A2 together with GST-Cdk2 or cyclin D1 or ΔC-cyclin D1 together with GST-Cdk4 with or without $p27^{Xic1}$, and the complex was purified as described (31). $p13^{Suc1}$ beads were produced as described previously (27).

Caspase Assays—For affinity labeling of active caspases (32), aliquots of an interphase extract treated with either buffer, cytochrome *c*, or ΔN-cyclin A2-Cdk2 were incubated with 1 μM biotinylated affinity reagent zEK(bio)D-aomk (Peptides International) for 10 min at 37 °C, resolved by 15% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Blots were stained with streptavidin-conjugated horseradish peroxidase (Calbiochem; 1:300 dilution) for 3 h at room temperature, and signals were detected by enhanced chemiluminescence. Cleavage of cyclin A2-Cdk2, cyclin A2-Cdk2-$p27^{Xic1}$, cyclin D1-Cdk4, and cyclin D1-Cdk4-$p27^{Xic1}$ was analyzed by incubating 250 ng of each complex with 15 units/μl of the indicated recombinant caspase (caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, BIOMOL Research laboratories). Reactions were incubated at 30 °C, and aliquots of 5 μl were withdrawn at the indicated times. Samples were separated by SDS-PAGE and immunoblotted with anti-cyclin antibody. Caspase-2, -3, and -7 activities were measured with the specific caspase colorimetric substrate QuantiPak™ from BIOMOL Research Laboratories using *N*-acetyl-VDEAD-*p*-nitroanilide, *N*-acetyl-DEVD-*p*-nitroanilide, and *N*-acetyl-DQMD-*p*-nitroanilide, respectively. For assays in crude extracts, embryos were homogenized in assay buffer, and samples corresponding to 3 embryos were used for each time point. Assay mixtures were incubated for 1 h at 22 °C before measurement of absorbance at 405 nm with a LabSystems MultiSkan MS microtiter plate reader. All measurements were repeated in triplicate for each time point, and the mean ± S.E. is reported.

In Vitro Kinase Assays—Phosphorylation of histones H2A, H2B, H3, H4 (Roche Molecular Biochemicals) by cyclin-Cdk complexes was carried out as described for histone H1 phosphorylation (27). Phosphorylation of the retinoblastoma protein (pRB) by cyclin D1-Cdk4 was carried out as described previously (21). A peptide encompassing Ser-32 of bovine histone H2B, (amino acids 27–34; KKRKRSRK) was synthesized by the HHMI Protein Chemistry Facility at the University of California, San Francisco. A phosphospecific antibody to Ser-32 in histone H2B was a kind gift of Dr. David Allis (University of Virginia). Phosphoamino acids were analyzed by two-dimensional electrophoresis at pH 1.9 and 3.5 on Kodak cellulose thin layer plates as described (33).

Isolation of Nuclei and Fluorescence-activated Cell Sorter Analysis—CHO-K1 cells were grown in 10-cm dishes with F12 medium containing 10% bovine serum (Invitrogen). Nuclei from ~10^7 cells were isolated essentially as described (34). For fluorescence-activated cell sorter analysis, nuclei (1000 nuclei/μl) were added to 100 μl of CSF-released extract and incubated for 1 h at room temperature before the addition of recombinant proteins. Samples were mixed with an equal volume of sucrose buffer (0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5% Nonidet P-40, 10 mM Tris-HCl, pH 8.0) for 10 min at room temperature, layered onto a sucrose cushion (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 8.0), and centrifuged for 45 min at 30,000 × *g* at 4 °C. The nuclei were resuspended by gentle vortexing in 200 μl of Krishan's stain and further dispersed by pipetting. Another 200-μl volume of Krishan's stain was added, and the samples were kept overnight at 4 °C. Flow cytometry was performed with a Coulter Epics-XL flow cytometer in the Flow Cytometry Core Facility at the University of Colorado Cancer Center.

RESULTS

Our previous results showed that G_1 arrest of the cell cycle occurs during prevention of apoptosis in embryos irradiated after the MBT. The G_1 arrest is a direct consequence of an increased level of $p27^{Xic1}$, 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 here whether cyclin-Cdk complexes are implicated in the induction or execution of apoptosis in *Xenopus* embryos irradiated before the MBT. Initially, cyclin A2-Cdk2 and cyclin D1-Cdk4 complexes were examined after irradiation *in vivo* because cyclins A1 and E are largely degraded after the MBT, and cyclin B-Cdc2 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.

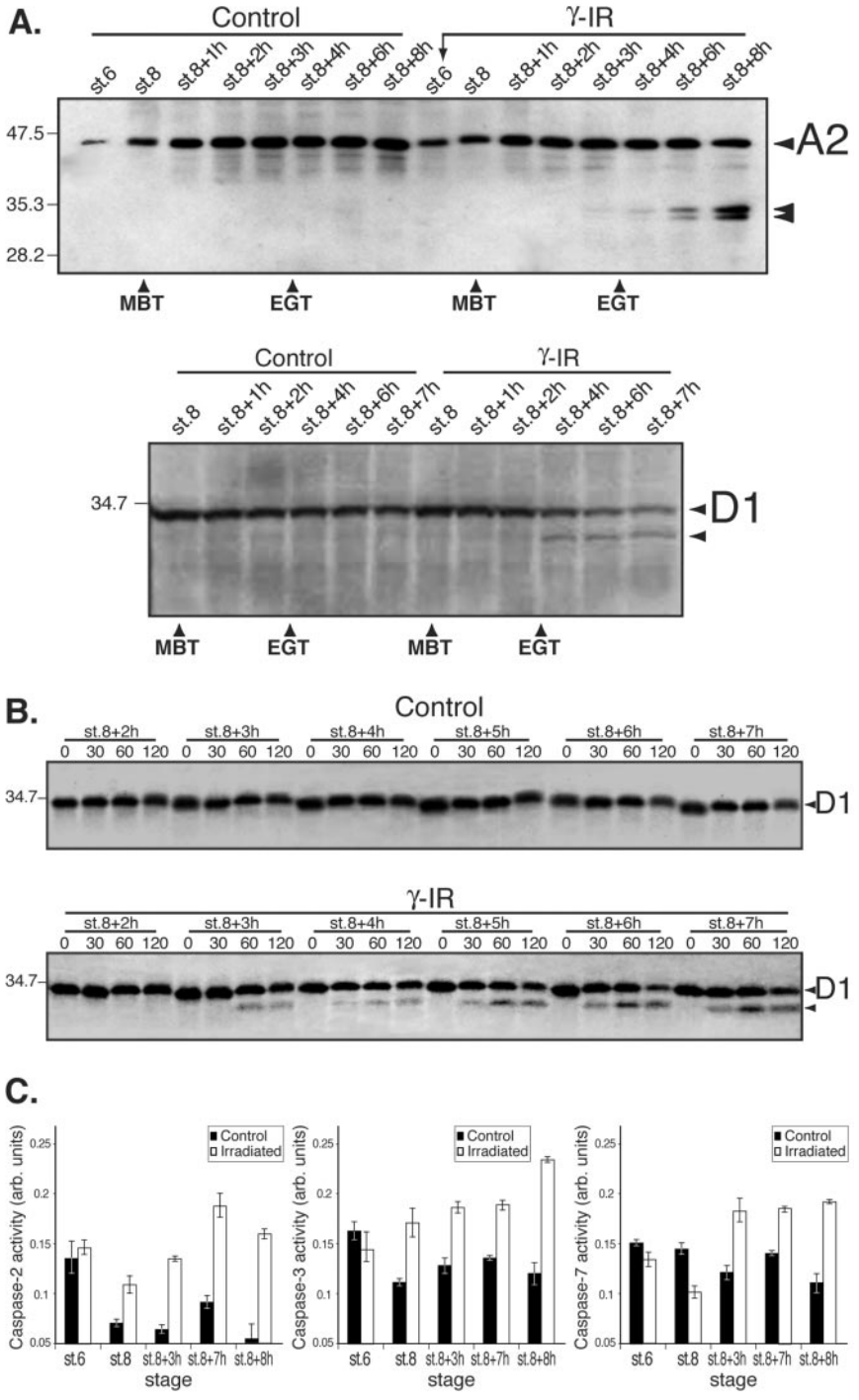

FIG. 1. **Cyclins A2 and D1 are cleaved during ionizing radiation-induced apoptosis.** Embryos were irradiated (*γ-IR*) or not (*Control*) before the MBT (*st.6*), collected at various times, and frozen. *A*, *upper panel*, samples equivalent to 15 embryos were precipitated with $p13^{Suc1}$ beads, and the bound proteins 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. 1*A*, *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 $p27^{Xic1}$ contributes to G_1 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. 1*A*, *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 [^{35}S]methionine-labeled cyclin D1 (Fig. 1*B*).

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. 1*C*) at the time when cyclin D1 and A2 cleavage occurs (Fig. 1*A*). 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. 1*A*), has a molecular weight identical to that observed after *in vitro* caspase cleavage (Fig. 2*D*). Interestingly, whereas the cleavage site of cyclin A2 has an Asp residue in the P4 position, the overall sequence (^{87}DEPD^{90}) does not represent a prototypical caspase-3 consensus sequence (37). This motif was changed to AEPD (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. 2*C*). 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. 2*D*, *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. 2*D*, *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 p27^{Xic1} 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. Structural studies indicate that p27^{Xic1} binds to both cyclin A and Cdk2 in the complex (38). This raises the question of whether caspases can cleave cyclins A2 and D1 when the complexes are associated with p27^{Xic1}. To assess this question, purified cyclin-Cdk and cyclin-Cdk-p27^{Xic1} complexes produced in baculovirus-infected insect cells were incubated with caspase-3 and tested for cyclin cleavage by immunoblotting. Caspase-3 is able to cleave cyclins A2 and D1 bound to their Cdk partner *in vitro* even when the complex is associated with p27^{Xic1} (Fig. 2*E*). Because p27^{Xic1} is not a caspase substrate,[2] the simplest interpretation of this result is that p27^{Xic1} does not protect the complex from caspase-mediated cleavage.

Cleavage of cyclin A2 at Asp-90 also removes the destruction

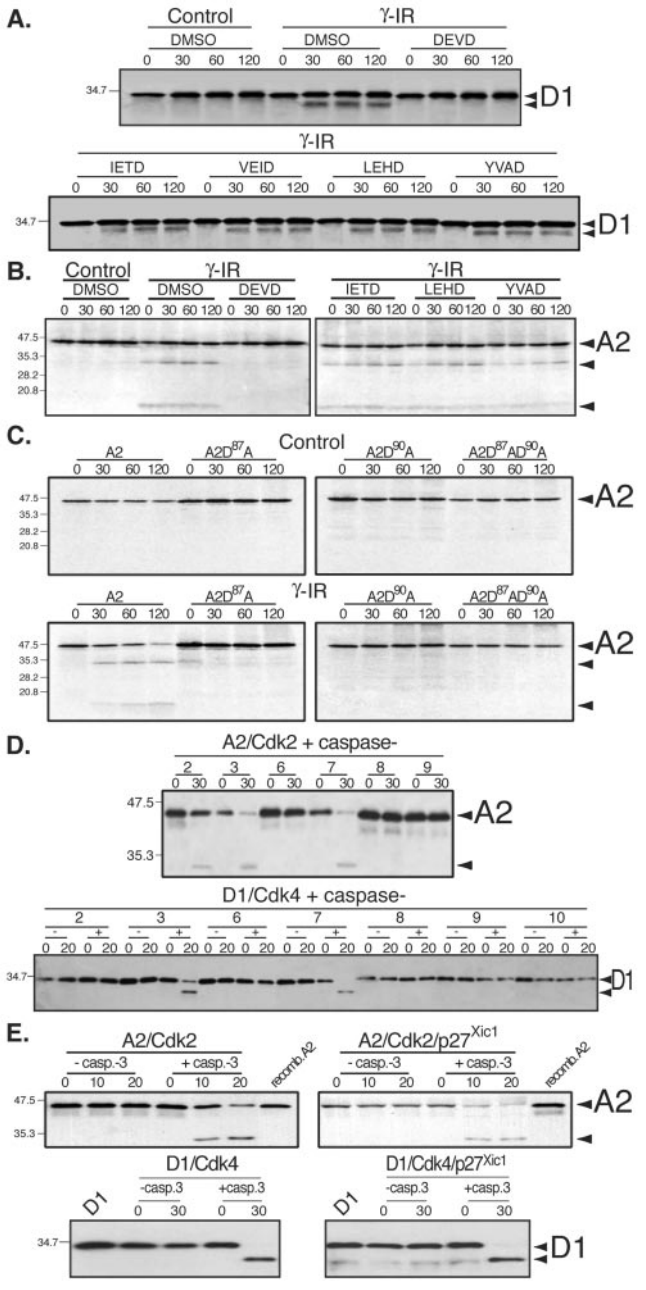

FIG. 2. **Cyclins A2 and D1 are substrates for caspase-mediated proteolysis.** *A*, extracts from non-irradiated embryos (*Control*) or embryos irradiated at stage 6 (*γ-IR*) and collected 6 h after the MBT were incubated with 250 nM of the indicated caspase inhibitor before the addition of radiolabeled cyclin D1. Ac-DEVD-CHO, Ac-IETD-CHO, Ac-VEID-CHO, Ac-LEHD-CHO, Ac-YVAD-CHO are aldehyde inhibitors of the subfamily of caspase-3, caspase-6 and -8, caspase-9, and caspase-1 respectively. Where indicated, Me_2SO (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 A2. *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-p27^{Xic1} (*upper panel*) or cyclin D1-Cdk4 and cyclin D1-Cdk4-p27^{Xic1} 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. 3*A*, *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. 3*A*, *middle panel*). In contrast, the level of ectopic ΔN-cyclin A2 remained constant throughout the time course analyzed (Fig. 3*A*, *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. 3*B* 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. 3*B*, *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. 3*C*). 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 $p13^{Suc1}$ beads to precipitate cyclin A2-Cdk2 after cleavage *in vitro* by caspase 3. Fig. 3*D* 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. 3*E*, 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. 4*A*). 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. 4*B*, *upper panel*) correlated with the presence of the cleaved form of cyclin D (Fig. 1*A*, *lower panel*) and did not result from an increased amount of endogenous $p27^{Xic1}$ (Fig. 4*B*, *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. 4*B*, *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-

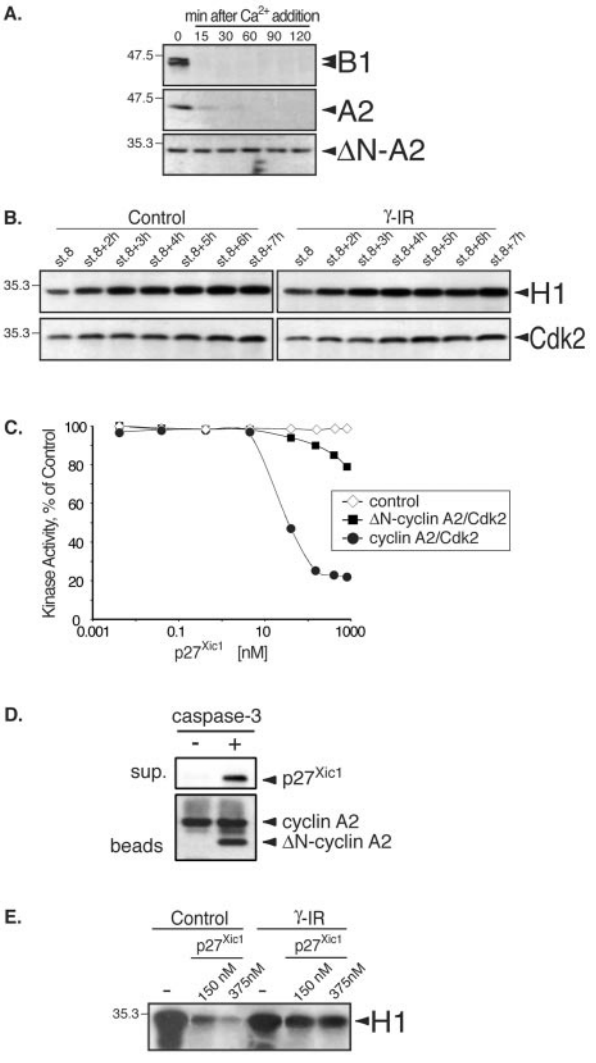

FIG. 3. **Caspase cleavage of cyclin A2 generates a truncated form with constitutive kinase activity.** *A*, a CSF-arrested extract was prepared as described under "Experimental Procedures" and released from metaphase arrest by calcium addition (*time 0*). To prevent reactivation of MPF by new cyclin B synthesis, 50 μg/ml cycloheximide was added with the calcium. Purified cyclin A2-Cdk2 or ΔN-cyclin A2-Cdk2 complexes (16 ng/μl) were added before calcium addition. Samples were taken at different times after calcium addition and analyzed for the levels of endogenous cyclin B1 and recombinant cyclin A2 and ΔN-cyclin A2 by Western blotting. *B*, embryos were not irradiated (Control) or irradiated before the MBT (γ-IR) and collected at various times. Samples were assayed for histone H1 kinase activity in cyclin A2 immunoprecipitates (*upper panel*). Aliquots of the immunoprecipitates were blotted for Cdk2 to confirm equivalent immunoprecipitation of cyclin A2 and ΔN-cyclin A2 (*lower panel*). *C*, equal amounts of purified cyclin A2-Cdk2 or ΔN-cyclin A2-Cdk2 complexes (50 ng) were preincubated with the indicated amounts of GST-$p27^{Xic1}$ or GST alone (*control*), and the kinase reaction was performed with histone H1 as substrate, as described under "Experimental Procedures." *D*, purified cyclin A2-Cdk2-$p27^{Xic1}$ from Sf9 cells was bound to $p13^{Suc1}$ beads and incubated with either recombinant caspase-3 (15 units/μl) or buffer (*control*) at 30 °C. After incubation, the supernatant (*sup.*) was withdrawn, and the beads were washed with 500 mM NaCl, 50 mM Tris-HCl, pH 8. Bound fractions were immunoblotted with anti-cyclin A2, and soluble fractions were immunoblotted with anti-$p27^{Xic1}$ antibodies. *E*, samples equivalent to 15 embryos from non-irradiated (*Control*) or embryos irradiated at stage 6 (*γ-IR*) were collected 7 h after the MBT and precipitated with $p13^{Suc1}$ beads for assay of endogenous cyclin A2-Cdk2 and ΔN-cyclin A2-Cdk2 activity. H1 kinase activity was assayed in the presence of the indicated concentrations of $p27^{Xic1}$.

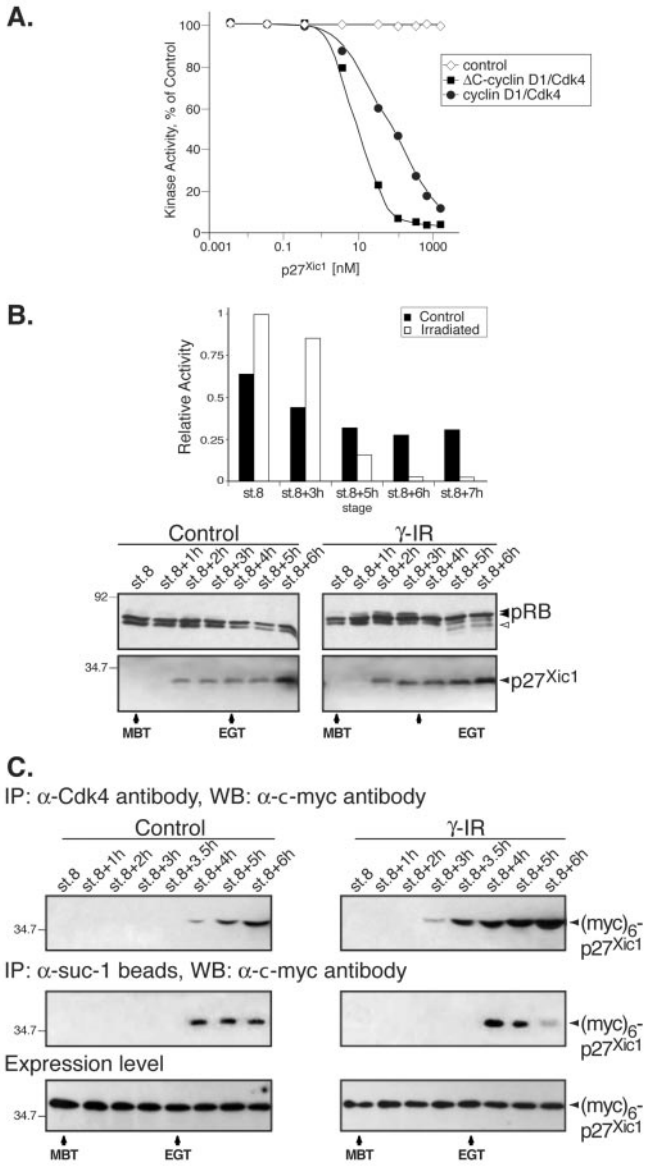

FIG. 4. **Caspase cleavage of cyclin D1-Cdk4 increases binding of and inhibition by $p27^{Xic1}$.** *A*, equal amounts of purified cyclin D1-Cdk4 or ΔC-cyclin D1-Cdk4 complexes from Sf9 cells (50 ng) were preincubated with the indicated amounts of GST-$p27^{Xic1}$ or GST alone (*control*), and the kinase reaction was performed with GST-pRB as substrate, as described under "Experimental Procedures." *B*, *upper panel*, samples equivalent to 15 embryos irradiated (*γ-IR*) or not (*Control*) before the MBT (*st.6*) were collected at the indicated times, precipitated with anti-Cdk4 antibody, and assayed for phosphorylation of GST-pRB *in vitro*. Samples from irradiated (*γ-IR*) or control embryos were blotted with anti-pRB and anti-$p27^{Xic1}$ antibodies (*middle* and *lower panels*). *Filled arrows* denote phospho/dephospho forms of pRB, and the *open arrow* denotes a proteolytically cleaved form of pRB. *C*, embryos were injected (50 nl) at the one-cell stage with *in vitro* transcribed and translated myc_6-$p27^{Xic1}$. Then embryos were irradiated (*γ-IR*) or not (*Control*) before the MBT (*st.6*) and collected at the indicated times, and the cyclin A2 and D1 complexes were precipitated (*IP*) with $p13^{Suc1}$ beads and anti-Cdk4 antibody, respectively. The level of $p27^{Xic1}$ associated with each complex was assessed by Western blotting (*WB*) using anti-c-myc antibody (*top* and *middle panel*). The total level of recombinant myc_6-$p27^{Xic1}$ remained constant throughout the experiment (*lower panel*) and was a negligible fraction of total $p27^{Xic1}$ in the embryo.

Cdk2 or cyclin D1-Cdk4 was assessed by immunoblotting the myc epitope on $p13^{Suc1}$ beads or Cdk4 immunoprecipitates, respectively. The results demonstrate that more $p27^{Xic1}$ is bound to cyclin D1-Cdk4 after irradiation, whereas a reduced amount of the inhibitor is detected after precipitation with $p13^{Suc1}$ beads, perhaps reflecting the reduced ability of $p27^{Xic1}$ to bind ΔN-cyclin A2-Cdk2 (Fig. 4*C*).

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 pycnotic 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. 5*A*, *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 G_1 level (sub-G_1 peak). Nuclear fragmentation induced in response to ΔN-cyclin A2-Cdk2 wt addition produced a characteristic sub-G_1 peak indicative of cleavage of the DNA (Fig. 5*A*, *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. 5*A*, *lower panel*). In addition, preincubation of the ΔN-cyclin A2-Cdk2 wt complex with $p27^{Xic1}$ did not abolish the apoptotic activity manifested in an interphase extract (Fig. 5*A*, *upper panel*), as expected, since $p27^{Xic1}$ is unable to inhibit ΔN-cyclin A2-Cdk2 activity (Fig. 3*C*). 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. 5*B*, *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. 5*B*, *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. 5*B*, *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 ΔN-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-

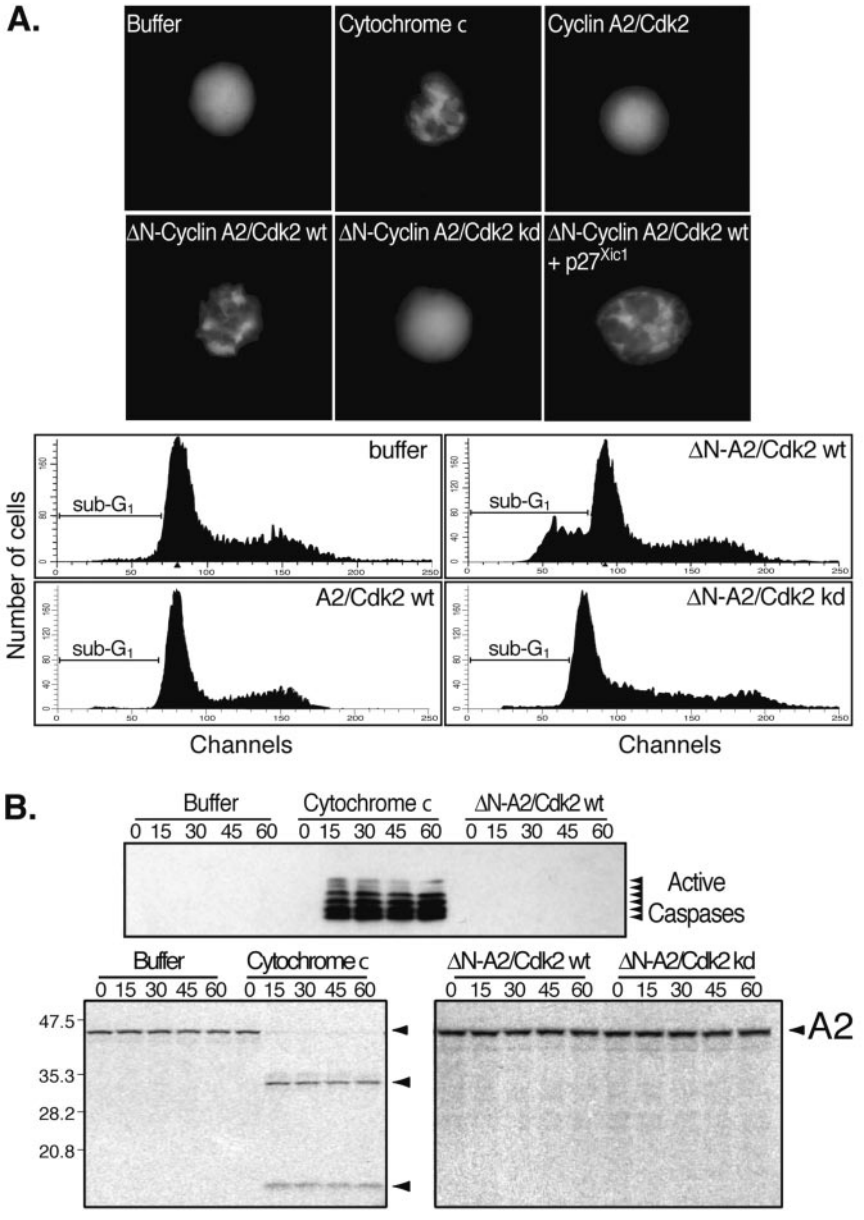

FIG. 5. **ΔN-cyclin A2-Cdk2 induces apoptotic nuclear morphology in *Xenopus* egg extracts.** *A*, *upper panel*, buffer, cytochrome *c*, purified cyclin A2-Cdk2, ΔN-cyclin A2-Cdk2 wt, ΔN-cyclin A2-Cdk2 kinase-dead (kd), or ΔN-cyclin A2-Cdk2 wt preincubated with $p27^{Xic1}$ were added to an interphase extract. After 30 min, 2-μl aliquots were fixed, stained with 4,6-diamidino-2-phenylindole, and visualized by fluorescence microscopy. *Lower panel*, interphase extracts were supplemented with CHO cell nuclei, treated with either buffer, cyclin A2-Cdk2, or ΔN-cyclin A2-Cdk2 (wt or kd) and analyzed by flow cytometry. *B*, *upper panel*, affinity labeling of active caspases. An interphase *Xenopus* egg extract was supplemented with either buffer, cytochrome *c*, or ΔN-cyclin A2-Cdk2 wt, and aliquots were removed at the indicated times, labeled with the zEK(bio)D-aomk caspase affinity reagent, subjected to SDS-PAGE, and visualized with peroxidase-coupled streptavidin. *Lower panel*, caspase-3 activity was also assessed by cleavage of the radiolabeled cyclin A2 in extracts containing either buffer, cytochrome *c*, or ΔN-cyclin A2-Cdk2 (wt or kd) as indicated. The addition of cytochrome *c* served as a positive control.

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. 6*A*). The activity of ΔN-cyclin A2-Cdk2 toward both histone H1 and H2B was inhibited by olomoucine (data not shown), a specific inhibitor of Cdks (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. 6*B*). 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 ($KKRKRS^{32}RK$). As shown in Fig. 6*C*, 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 $p13^{Suc1}$ beads. H2B kinase activity was detected only in apoptotic embryos and was correlated with the presence of the cleaved form of cyclin A2 (Fig. 6*D*). 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 phospho-specific antibody. Ser-32 phosphorylation occurred only when ΔN-cyclin A2-Cdk2 was present (Fig. 6*E*). Moreover, all activity against Ser-32 in H2B could be depleted by $p13^{Suc1}$ beads (data not shown), suggesting ΔN-cyclin A2-Cdk2 is the enzyme responsible for Ser-32 phosphorylation in the extract.

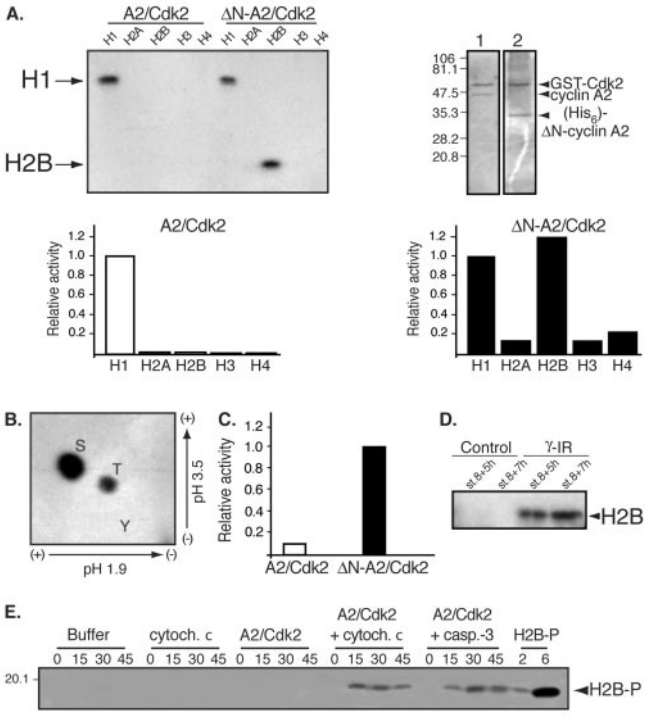

FIG. 6. **ΔN-cyclin A2-Cdk2 exhibits expanded substrate specificity.** *A*, *upper panel*, substrate specificity of cyclin A2-Cdk2 and ΔN-cyclin A2-Cdk2 was assessed with histones H1, H2A, H2B, H3, and H4. In these experiments the amount of each cyclin-Cdk preparation used was adjusted such that both complexes showed similar histone H1 kinase activity. An aliquot (2 μl) of each kinase reaction was analyzed by SDS-PAGE and autoradiography (*left panel*). On the *right* is a stained gel of the cyclin A2-Cdk2 (*lane 1*) and ΔN-cyclin A2-Cdk2 (*lane 2*) preparations. *Lower panel*, histone kinase activity of cyclin A2-Cdk2 (*left*) and ΔN-cyclin A2-Cdk2 (*right*) was quantified as described under "Experimental Procedures" and normalized to H1 activity, which is indicated as 1.0. *B*, phosphoamino acid analysis of histone H2B phosphorylated by ΔN-cyclin A2-Cdk2. *S*, serine; *T*, threonine; *Y*, tyrosine. *C*, phosphorylation of the KKRKRS^{32}RK synthetic peptide by either cyclin A2-Cdk2 or ΔN-cyclin A2-Cdk2 with activity by the latter complex set as 1.0. *D*, samples equivalent to 15 embryos from non-irradiated (*Control*) or embryos irradiated at stage 6 (*γ-IR*) and collected 5 and 7 h after the MBT were precipitated with p13^{Suc1} beads for endogenous cyclin A2-Cdk2 and ΔN-cyclin A2-Cdk2. Kinase activity was assayed using histone H2B as substrate, and an autoradiograph is shown. *E*, a CSF-arrested extract was released by calcium addition (*time 0*) and maintained in interphase with 50 μg/ml cycloheximide as described under "Experimental Procedures." Protein kinase inhibitors (protein kinase A inhibitor (*PKI*; 10 μM), protein kinase C inhibitor (pseudosubstrate peptide encoding amino acids 19–36; 10 μM), mitogen-activated protein kinase kinase inhibitor (U0126; 50 μM), and histone H2B (5 mg/ml) were added to the extract and incubated for 15 min at room temperature. Buffer, cytochrome *c*, and cyclin A2-Cdk2 (16 ng/μl) alone or in combination with cytochrome *c* or caspase-3 (15 units/μl) were added, and samples were taken at different times and analyzed for Ser-32 phosphorylation of histone H2B using a phospho-specific antibody. Phospho (*P*)-histone H2B (2 and 6 μg) prepared *in vitro* using ΔN-cyclin A2-Cdk2 was also loaded as positive control.

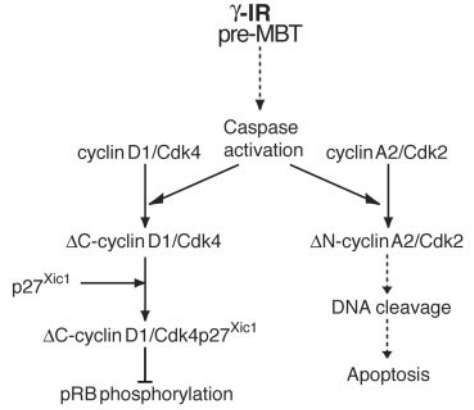

FIG. 7. **Model of G_1/S Cdk regulation during apoptosis.** Ionizing radiation administered before the MBT promotes apoptosis by activating a caspase cascade that leads to cleavage of cyclins A2 and D1. The ΔN-cyclin A2-Cdk2 complex is constitutively activated and through phosphorylation of histone H2B might promote nucleosomal cleavage of DNA. The ΔC-cyclin D1-Cdk4 complex may sequester p27^{Xic1} and lead to reduced phosphorylation of pRB during apoptosis.

DISCUSSION

In *Xenopus* embryos, an apoptotic program is abruptly activated after the MBT if the pre-MBT embryo experienced DNA damage or was treated with inhibitors of transcription, translation, or DNA replication (18–20, 29, 48). Our results indicate that the hallmarks of apoptosis are detected only at the onset of gastrulation and correlate most strongly with activation of the caspase-3 subfamily (Fig. 1*B*), suggesting this is the major executioner caspase activated during ionizing radiation-induced apoptosis. The data presented in this paper show that the induction of specific cyclin cleavage by caspase-3 to generate ΔC-cyclin D1-Cdk4 and ΔN-cyclin A2-Cdk2 is an early step during apoptosis in *Xenopus* embryos. Previous data in other 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 p27^{Xic1} 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 ΔC-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 ΔC-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. 2*A*), (ii) ΔN-cyclin A2 and ΔC-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 Asp-87 and Asp-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 ΔN-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 ΔN-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 ΔpRB, 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 ΔpRB (Fig. 4*B*, *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 ΔN-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 DNAses. At least two parallel and redundant pathways are known that can lead to nuclear apoptosis. One of these pathways involves a caspase-activated DNase (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 ΔN-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 ΔN-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 ΔN-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 Cδ 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 ΔN-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 ΔN-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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