Circulation Research
Journal of the American Heart Association

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Circ Res. 2002;91:782-789; originally published online October 10, 2002;
doi: 10.1161/01.RES.0000041030.98642.41
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Inhibition of Hypoxia-Induced Apoptosis by Modulation of Retinoblastoma Protein–Dependent Signaling in Cardiomyocytes

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Abstract—Apoptotic cell death is an important mode of cell loss contributing to heart dysfunction. To analyze the importance of the E2F-dependent regulation of gene transcription in cardiomyocyte apoptosis, the function of cell cycle factors impinging on the retinoblastoma protein (pRb)/E2F pathway was investigated. In isolated neonatal ventricular myocytes, apoptotic cell death induced by hypoxia (deferoxamine, 100 μmol/L) specifically activated cyclin-dependent kinases (cdks) 2 and 3. Apoptotic cell death was inhibited by ectopic expression of cdk inhibitors $p21^{CIP}$ and $p27^{KIP1}$ but not $p16^{INK4}$. In addition, apoptosis was also abrogated by forced expression of kinase dead mutant proteins of cdk2/3 but not of cdk4/6. Introduction of cdk inhibitors or dominant-negative cdk2/3 blocked pRb hyperphosphorylation and abrogated E2F-dependent gene transcription, including that of the E2F-responsive genes of proapoptotic caspase 3 and caspase 7. Moreover, introduction of constitutively active pRb and transcriptionally inert mutant E2F1/DP1 efficiently protected cardiomyocytes from apoptosis. In conclusion, these data demonstrate that cdk-specific inactivation of pRb and the subsequent activation of E2F-dependent gene transcription are required for cardiomyocyte apoptosis. **(*Circ Res*. 2002;91:782-789.)**

Key Words: apoptosis ■ hypoxia ■ cell cycle ■ signaling

Apoptotic cell death is a universal mechanism for multicellular organisms to regulate appropriate growth during development, tissue homeostasis, and toxic stress through the elimination of cells.[1] Previous studies have shown that hypoxia is a sufficient trigger for cardiac myocyte apoptotic cell death.[2–4] In addition, cardiomyocytes also undergo extensive programmed cell death in response to ischemic injury in vivo.[4] Therefore, loss of cardiac cell mass by apoptosis during myocardial ischemia constitutes a crucial process substantially contributing to heart failure.

Shortly after birth, cardiomyocytes exit the cell cycle and persist in a cell cycle stage defined as G_0.[5] These differentiated cardiac myocytes lose their ability to divide but retain the ability to grow through enlargement of cell size in response to a variety of mitogenic stimuli. Progression through the mammalian cell cycle is tightly regulated by the activation of cyclin-dependent kinases (cdks). The enzymatic activity of cdks is positively regulated through association with cyclins and negatively regulated by cdk inhibitors (cdkis) of the INK4 and KIP families.[5] The transition from G_0 to G_1 phase is regulated by cdk4/6 and D-type cyclins, whereas cdk2/cyclin E/A and cdk3 control the progression from the G_1 phase into the S phase. Whereas $p16^{INK4}$ specifically inhibits cdk4/6 complexes, $p21^{CIP1}$ and $p27^{KIP1}$ reportedly bind to all of these G_1 cdks. Retinoblastoma protein (pRb) is inactivated through subsequent phosphorylation by cdk4/6 and cdk2/3 during mid to late G_1 phase.[6–8] Hypophosphorylated active pRb carries an E2F/DP heterodimer (hereafter designated as E2F), resulting in transcriptional repression of E2F target genes. Inactivation of pRb by hyperphosphorylation releases E2F, which is now transcriptionally active.[9] Targeted inactivation of the E2F1 gene revealed a dual role for E2F1 in the control of cellular growth and apoptosis in vivo, reflecting the capacity of pRb/E2F1 complexes to repress both genes involved in apoptosis and genes necessary for DNA synthesis.[10]

More recent studies have suggested a critical role for cdks and cdkis during hypertrophic growth of cardiac myocytes.[5] We and others have shown that ectopic expression of E2F1 results in cell cycle reentry and DNA synthesis in cardiomyocytes.[11–13] Recently, the requirement for cdk2/cyclin A signaling was shown to be essential for cardiomyocyte apoptosis induced by hypoxia.[14] Collectively, these data imply a role of the cell cycle machinery in cardiomyocyte apoptosis. However, the functions of the targeting of cdks by cdkis and the downstream effector molecules of cdks and their involvement in the regulation of apoptosis of injured heart muscle cells are unknown. The present study examined the impact of hypox-

Original received May 7, 2001; resubmission received July 12, 2002; revised resubmission received September 5, 2002; accepted September 23, 2002.

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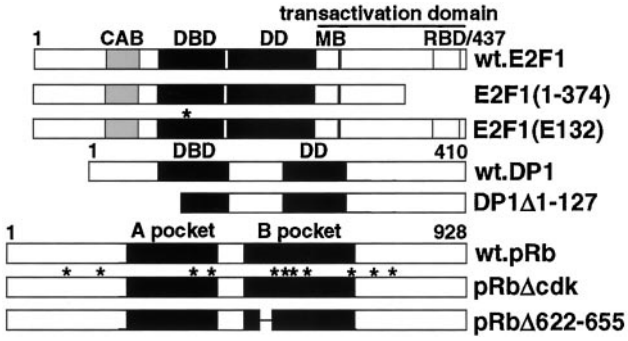

Figure 1. Schematic overview of adenoviral constructs used in the present study. CAB indicates cyclin A binding domain; DBD, DNA binding domain; DD, dimerization domain; MB, marked box; RBD, pRb binding domain; and asterisks, sites of mutations. E2F1Δ24 carries a deletion of the CAB site located at the N-terminal.[39] In E2F1(1-374), the transactivation domain has been deleted.[39] E2F1(E132) is DNA-binding deficient because of a point mutation within its DBD.[39] Dimerization-defective DP1Δ1-127 renders E2F/DP complexes transcriptionally inert.[15] Constitutively active pRbΔcdk carries point mutations in 11 cdk phosphorylation sites, rendering it refractory to cdk-dependent phosphorylation.[40] pRbΔ622-655 carries a deletion in the B-pocket domain and thus does not bind to E2F.[16]

ia-induced apoptosis on cell cycle–regulatory factors in cardiomyocytes. We show that under oxygen deprivation, cardiomyocyte apoptosis selectively activates cdk2/3, leading to the inactivation of pRb and induction of E2F-dependent gene transcription. Under these conditions, apoptosis was inhibited by ectopically expressed $p21^{CIP1}/p27^{KIP1}$, but not $p16^{INK4}$, and by dominant-negative cdk (dn.cdk) 2/3. Importantly, cardiac myocytes could be rescued from hypoxia-induced cell death by dominant-negative pRb and transcriptionally inactive E2F1/DP1.

Materials and Methods

Isolation of Rat Primary Cardiomyocytes

Ventricular cardiomyocytes from 4-day-old postnatal Wistar rats were isolated as described, except that cell suspensions were preplated twice and held in serum-free medium in the presence of 10 μmol/L cytosine arabinoside (Sigma Chemical Co) for 48 hours.[11] Cultures contained 3% to 8% noncardiomyocyte cells as routinely monitored in parallel by indirect immunofluorescence staining with monoclonal antibody to sarcomeric myosin heavy chain (Mab1628, Chemicon).

Recombinant Adenoviral Constructs and Transfections

Ad-$p16^{INK4}$, Ad-$p21^{CIP1}$, Ad-β-Galactosidase (β-Gal), and pcDNA3.1-wt.pRb were supplied by Carsten Brand; pcDNA3.1-wt.E2F1 was from Martin Lipp (both from the Max Delbrück Center). Ad-$p27^{KIP1}$ was obtained from Perry Nisen (Anderson Cancer Center). E2F1Δ24, E2F1(E132), and E2F1(1-374) were provided by Karen Vousden (National Cancer Institute, Frederick, Md). Ad-pRbΔcdk was from DeveloGen. Construction of pRbΔ622-655 and DP1Δ1-127 was performed as described.[15,16] An overview of these constructs is given in Figure 1. The dn.cdks were generated with synthetic oligonucleotides carrying the desired mutation and a Site Directed Mutagenesis Kit (Stratagene), as described previously.[8,17] Recombinant adenovirus containing a cytomegalovirus immediate-early gene promoter driving wild-type (wt) or mutant (mt) human cDNAs was generated using the AdEasy Adenoviral Vector System according to the manufacturer's instructions (Stratagene). Virus propagation, purification, and titering were performed as described.[11] At 6 hours after adenoviral infection (100 plaque-forming units [pfu]/cell, unless stated otherwise), cardiomyocytes were exposed to deferoxamine mesylate (DFx, 100 μmol/L; lot B28156, Calbiochem) for 20 hours, which served as an established hypoxia model system.[18,19] The expression and nuclear localization of ectopically expressed proteins were confirmed by fluorescence microscopy of fixed cells using immunostaining with specific antibodies (data not shown).

Reporter Gene Assays

All transfections were performed after adenoviral infection using the cationic liposomal transfection reagent DAC-30 (Regina Rezka, Max-Delbrück Center). Cardiomyocytes were seeded at a density of 8×10^5 cells on 24-well plates. Adherent cells were transfected with 3 μg of either pI-H2A68 or pI-H2A68* (Martin Lipp, Max-Delbrück Center), E2F1Luc (wt) and E2F1Luc (E2F−) (Joseph Nevins, Duke University Medical Center), and 0.5 μg β-Gal. pI-H2A68 carries the E2F consensus binding site of the human histone H2A promoter, which was mutated in pI-H2A68*. The E2F1Luc (wt) promoter construct contains several E2F binding sites fused to a luciferase reporter gene, which were mutated in E2F1Luc (E2F−).[20] At 20 hours after infection, luciferase activity was determined as described.[21] To correct for transfection efficiencies, the activity of β-Gal in the lysate was measured using standard methods. Transfection efficiency was between 2% to 7%, as evaluated by cotransfection of 0.5 μg of a cytomegalovirus-driven GFP expression plasmid (pEGFP-N1, Clontech).

Preparation of Cellular Extracts, Immunoblotting, and Protein Kinase Assays

Cell lysis, determination of protein content, Western blotting, and immune complex kinase assays were performed as described.[11] The following antibodies were used: cdk2 (sc-163), cdk3 (sc-826), $p16^{INK4}$ (sc-1207), $p21^{CIP1}$ (sc-471), $p27^{KIP1}$ (sc-528), and pRb (sc-50) (all from Santa Cruz); cdk4 (RB-026-P0), cdk6 (MS-398-P0), DP1 (RB-1443-P0), and E2F1 (RB-027-P0) (all from NeoMarkers); and GFP (rabbit polyclonal, from Clontech).

Flow Cytometry, Immunofluorescence, and In Situ Apoptosis Assay

Flow cytometry, immunostaining, and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) assay of cardiomyocytes immobilized on coverslips were performed as described previously.[11]

Caspase 3 Assay and Northern Blotting

Caspase 3 activity was measured according to the manufacturer's instructions (Oncor), and Northern blotting of total RNA was performed as decribed.[21]

Results

Differential Behavior of Cyclin-Dependent Kinases in Hypoxia-Induced Cardiomyocyte Apoptosis

To assess their role in cardiomyocyte apoptosis, the expression and phosphotransferase activity of endogenous and ectopically expressed cdks were determined in cardiomyocytes infected with wt.cdks or dn.cdks in the presence or absence of DFx. Hypoxia resulted in an almost complete downregulation of endogenous cdk4/6 protein levels, whereas cdk2/3 expression remained unaffected (Figure 2A). Notably, in the presence of DFx, the phosphotransferase activity of endogenous cdk2/3 was significantly induced, whereas no enzymatic activity was measured in anti-cdk4/6 immunoprecipitates. Furthermore, ectopic expression of dn.cdk2/3, but not dn.cdk4/6, antagonized DFx-dependent cardiomyocyte

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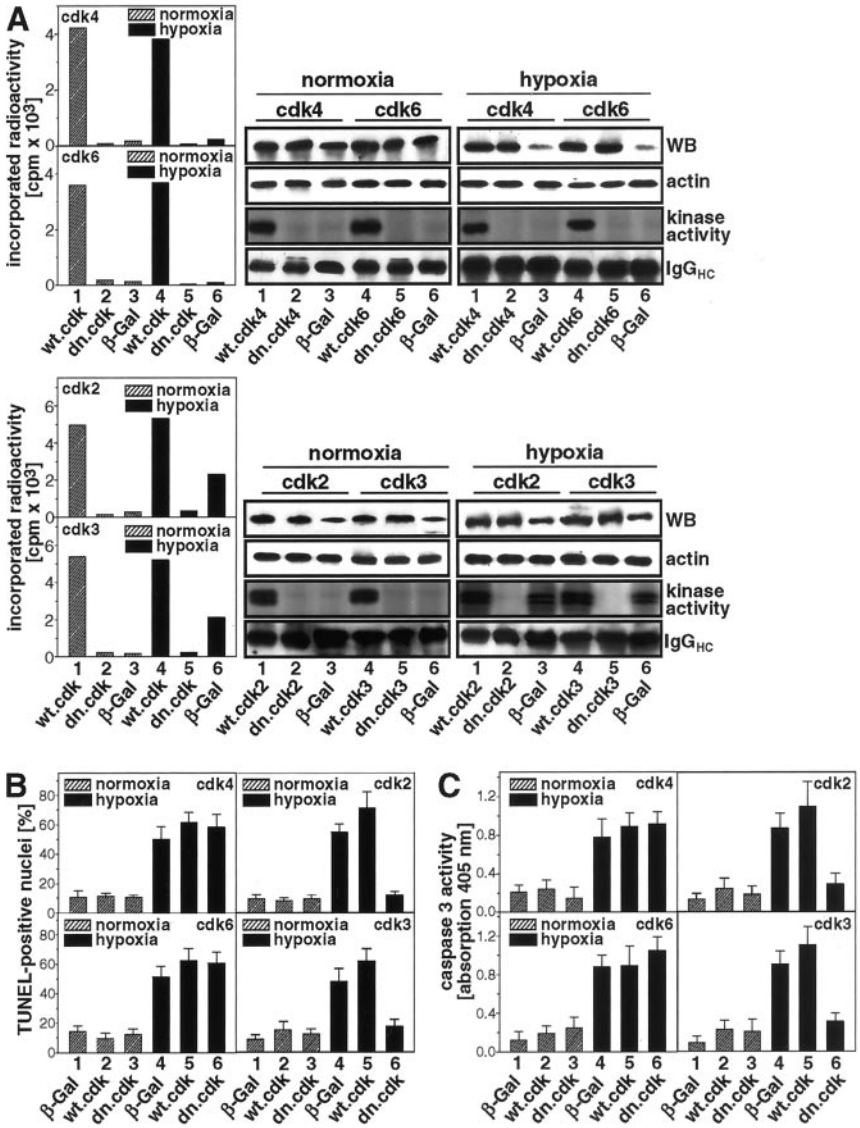

Figure 2. Inhibition of hypoxia-induced apoptosis by catalytically inactive dn.cdk in cardiomyocytes. Four-day-old neonatal rat ventricular cardiomyocytes were infected with recombinant adenoviral constructs (100 pfu/cell), and apoptosis was induced by addition of DFx (100 μmol/L) for 20 hours. A, Protein expression and immune complex kinase activities of cdks in cardiomyocytes. WB indicates Western blot. Protein expression was analyzed by resolving total protein extracts corresponding to 2×10^5 cells per lane by denaturing SDS-PAGE. Equal loading of bands was confirmed by reprobing with anti-sarcomeric actin antibody. Kinase assays were performed by incubation of cellular extracts (500 μg total protein cdk2/4/6 and 2.0 mg total protein cdk3) with cdk-specific antibodies as indicated. Kinase activities were measured using either histone H1 (cdk2 and cdk3) or recombinant pRb (cdk4 and cdk6) as substrates. The amount of incorporated radioactive label was analyzed after SDS-PAGE in a PhosphorImager using TINA software. Representative results of at least 2 independent experiments are shown. B, Apoptotic cell death detected using TUNEL of fragmented nuclear DNA and FITC-conjugated anti-digoxigenin antibody (green). A total of 200 nuclei in a randomly selected field were counted by fluorescence microscopy. C, Colorimetric assay for determination of caspase 3 activity in apoptotic cardiomyocytes. Mean±SD values of 3 independent experiments are shown.

apoptosis (Figures 2B and 2C). In conclusion, hypoxia-induced cardiomyocyte apoptosis is characterized by selective activation of cdks 2 and 3.

$p21^{CIP1}$ and $p27^{KIP1}$ but Not $p16^{INK4}$ Rescue Cardiomyocytes From Apoptosis

The cdkis $p16^{INK4}$, $p21^{CIP1}$, and $p27^{KIP1}$ are all expressed in cardiomyocytes under normoxic culture conditions (Figure 3A). However, exposure to DFx led to the downregulation of all 3 cdkis. Neither the in vitro kinase activity of immunoprecipitated cdk2/3 derived from hypoxic cardiomyocytes nor the induction of apoptosis in these cells was affected by ectopic expression of $p16^{INK4}$ (Figures 3A through 3C). In contrast, ectopic $p21^{CIP1}$ or $p27^{KIP1}$ effectively blocked both cdk2/3 activity and hypoxia-induced apoptosis in cardiomyocytes. Collectively, these data suggest that downregulation of specific cdkis with the subsequent activation of a defined subset of their target cdks is required for hypoxia-induced cardiomyocyte apoptosis.

$p21^{CIP1}$ and $p27^{KIP1}$ Exert cdk-Independent Antiapoptotic Effects in Cardiomyocytes

To elucidate whether the antiapoptotic effect of $p21^{CIP1}$ and $p27^{KIP1}$ is related to their inhibitory effect on cdk activity, wt.cdks were expressed in the presence of ectopic cdkis in DFx-stimulated cardiomyocytes, and the reinduction of apoptosis was monitored (Figure 4). In the presence of ectopic cdkis ($p16^{INK4}$, $p21^{CIP1}$, and $p27^{KIP1}$), coexpression of wt.cdks (cdk2/3 and cdk4/6) could restore kinase activity in cdk immune precipitates (Figure 4, bottom panel). However, only in the presence of $p16^{INK4}$ could reinduction of apoptosis be observed (Figure 4, top and middle panels). Moreover, in the presence of dn.cdk2/3, only the expression of the cognate wt counterpart could reestablish DFx-dependent apoptosis (Figure 4), indicating that both cdk2 and cdk3 kinase activities are required for hypoxia-induced cardiomyocyte apoptosis. Taken together, these data indicate that $p21^{CIP1}$ and $p27^{KIP1}$ exert their antiapoptotic effect through additional mechanisms unrelated to cdk inhibition.

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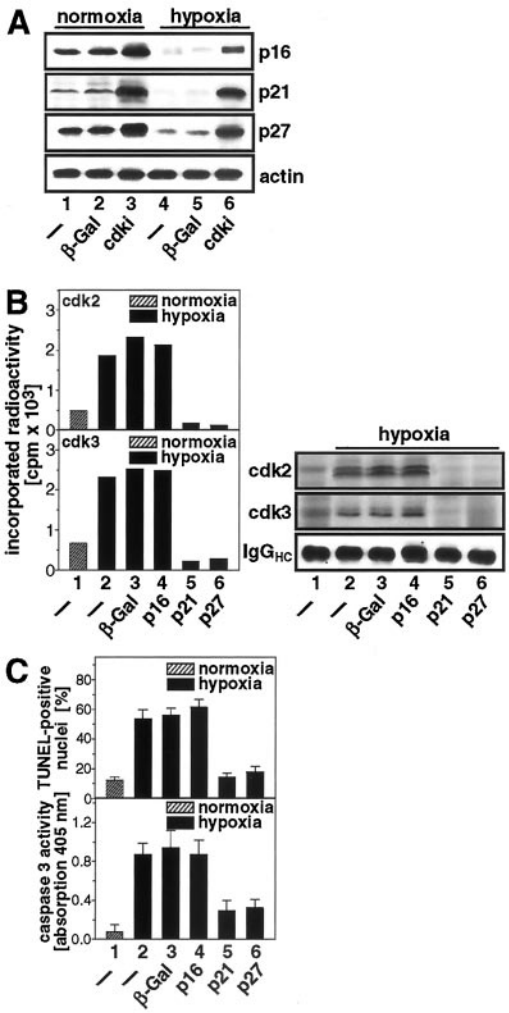

Figure 3. Inhibition of hypoxia-mediated apoptosis by cdkis. A, Western blot analysis shows the expression of endogenous and ectopically expressed cdkis in cardiomyocytes. Cellular extracts corresponding to 1.5×10^5 cells were subjected to SDS-PAGE and probed with specific antibodies as indicated. B, Ectopic $p21^{CIP1}/p27^{KIP1}$ but not $p16^{INK4}$ inhibits the activation of cdk2/3 during hypoxia-induced cell death. In vitro kinase activities of cdk2/3 were measured in cardiomyocyte cellular extracts as described in the Figure 2 legend. Representative results of at least 2 independent experiments are shown. C, Assessments of apoptotic cell death by TUNEL assay and caspase 3 activity in cardiomyocytes are shown. Mean±SD values of 3 independent experiments are shown.

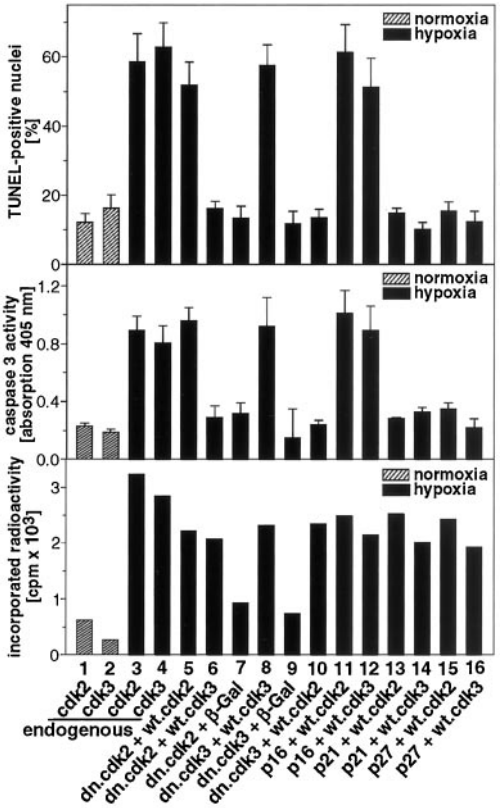

Figure 4. Antiapoptotic effect of $p21^{CIP1}$ and $p27^{KIP1}$ is independent of their cdk-inhibitory function. TUNEL assay (top), caspase 3 activation (middle), and immune complex kinase assays (bottom) were performed as described in the Figure 2 legend. Each viral construct was used at 50 pfu. Lanes 1 through 4 depict the endogenous kinase activities of β-Gal–infected control cardiomyocyte cultures.

cdk2/3-Dependent Inactivation of pRb Occurs in Cardiomyocyte Apoptosis

pRb is a well-known substrate of cdks.[7] Cell lysates from infected cardiomyocytes were subjected to denaturing gel electrophoresis, and pRb was immunodetected with an pRb-specific polyclonal antibody recognizing the hypophosphorylated and hyperphosphorylated isoforms. Under normoxic culture conditions, pRb protein was visualized as a single band representing the hypophosphorylated growth-suppressive form (Figure 5A, lane 1). In DFx-stimulated cardiomyocytes, a second pRb protein variant with a lower electrophoretic mobility corresponding to the inactivated hyperphosphorylated pRb variant was detected (Figure 5A, lanes 2 and 3). This inactivating phosphorylation of pRb was substantially diminished in the presence of ectopic dn.cdk2/3 or $p21^{CIP1}/p27^{KIP1}$ (Figure 5A, lanes 5 through 8). In contrast, the forced expression of dn.cdk4/6 or $p16^{INK4}$ failed to prevent the appearance of hyperphosphorylated pRb (Figure 5A, lanes 4, 9, and 10). These data indicate that in cardiomyocytes hypoxia leads to the inactivation of pRb via cdk2/3-dependent phosphorylation.

Functional Inactivation of pRb With Subsequent Activation of E2F1/DP1 Complexes Is Required in Cardiomyocyte Apoptosis

The phosphorylation status of pRb controls the activity of the E2F1/DP1 heterodimer transcription factor complex with which pRb interacts.[1] Therefore, wt or various mt constructs of pRb, E2F1, and DP1 (see Figure 1) were ectopically expressed in cardiomyocytes, and their cellular effects on cardiomyocyte survival were monitored by TUNEL assay and detection of caspase 3 activity (Figure 5B) or by flow cytometric determination of apoptotic cells residing in the sub-G_1 population (data not shown). Constitutively active pRbΔcdk effectively blocked hypoxia-induced cardiomyocyte apoptosis. In contrast, pRbΔ622-655, deficient for E2F binding, was ineffective in this regard. These data show that phosphorylation-mediated functional inactivation of pRb is

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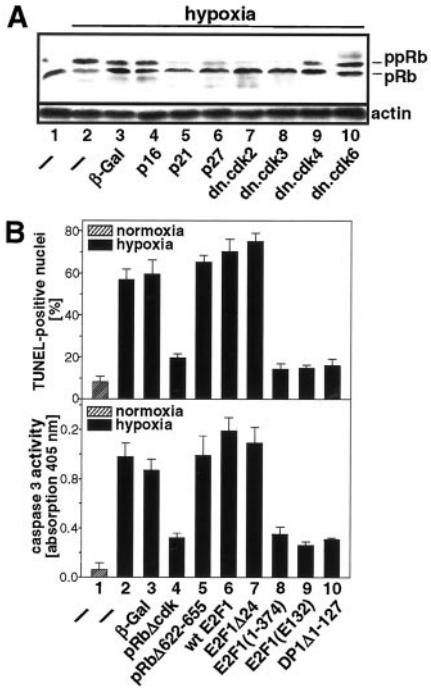

Figure 5. A, Inhibition of pRb phosphorylation by overexpression of dn.cdk2/3 and $p21^{CIP1}/p27^{KIP1}$. Cellular extracts corresponding to 1.5×10^5 cells were subjected to SDS-PAGE and probed with an pRb-specific antibody. One representative blot of 4 to 6 independent experiments is shown. B and C, Determination of apoptotic cell death by TUNEL assay (B) and caspase 3 activity (C). Mean±SD values of 3 independent experiments are shown.

required for hypoxia-induced cardiomyocyte apoptosis. In contrast to wt.E2F1 and E2F1Δ24, transcriptionally inactive E2F1(1-374) and E2F1(E132) were able to rescue cardiomyocytes from apoptosis (Figure 5B). Moreover, the expression of DP1Δ1-127, abrogating endogenous E2F DNA binding activity, also showed a marked repression of cardiomyocyte apoptosis (Figure 5B). We infer from these data that E2F-mediated transactivation capacity is required for cardiomyocyte apoptosis.

Hypoxia-Induced Apoptosis Involves Both Transcriptional Activation and Derepression of E2F-Regulated Promoters in Cardiomyocytes

In the next set of experiments, the capacity of endogenous E2F to transactivate E2F-responsive promoters during hypoxia was investigated. Exposure to DFx led to marked induction of histone H2A gene transcription in cardiomyocytes (Figure 6A). This increase in reporter activity was specific to E2F, inasmuch as (1) no such effect was observed for pI-H2A-68* with a mutated E2F-site, and (2) ectopic expression of E2F1(E132), E2F1 (1-374), DP1Δ1-127, dn.cdk2/3, and pRbΔcdk but not pRbΔ622 to 655 inhibited the luciferase reporter significantly (Figure 6A). These data imply that proapoptotic processes in cardiomyocytes recruit E2F-dependent transcriptional activation.

We now analyzed whether E2F binding sites were transcriptionally derepressed in apoptotic cardiomyocytes. Previously, the E2F1 gene promoter was shown to be repressed in

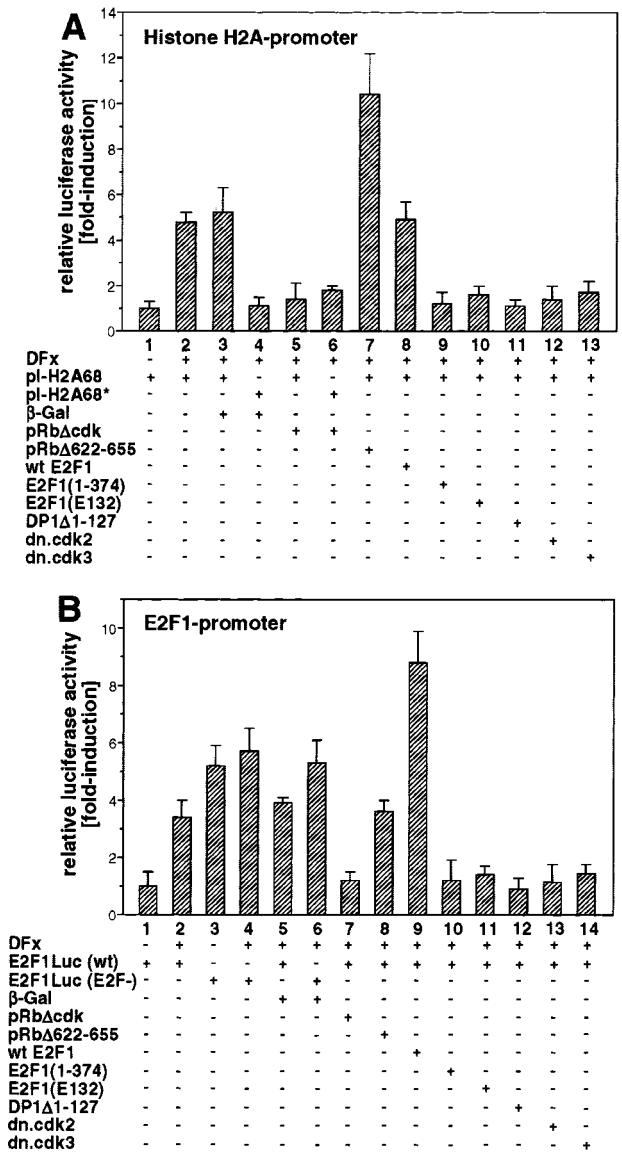

Figure 6. Regulation of E2F-dependent promoters during hypoxia-triggered cardiomyocyte cell death. A, Induction of apoptosis leads to E2F-dependent gene transcription in cardiomyocytes. The E2F-responsive histone H2A.1 luciferase reporter construct was transfected alone or in combination with various mutated pRb/E2F1/DP1 constructs. pI-H2A carries a wt.E2F consensus DNA binding site, whereas this site is mutated in pI-H2A-68*. B, Derepression of E2F-regulated gene transcription in apoptotic cardiomyocytes is shown. The E2F1Luc (wt) contains a 728-bp fragment of the human E2F1 promoter fused to a luciferase reporter gene. In E2F1Luc (E2F−), the E2F1 sites in the same E2F1 promoter fragment have been mutated. Luciferase activity was determined from whole-cell lysates (60 μg total protein) and corrected for β-Gal activity. Mean±SD values of 3 independent experiments are presented.

G_0 in an E2F1-independent fashion, whereas it is derepressed in G_1/S in an E2F-dependent fashion.[20] Under normoxia, the reporter E2F1Luc (E2F−) with mutated E2F sites displayed a 5.2-fold induction of luciferase activity compared with E2F1 (wt) with intact E2F sites (Figure 6B). These results indicate that E2F-responsive genes are under transcriptional repression in unstimulated cardiomyocytes, which is in agreement with the lack of endogenous cdk activities (Figure 2).[9]

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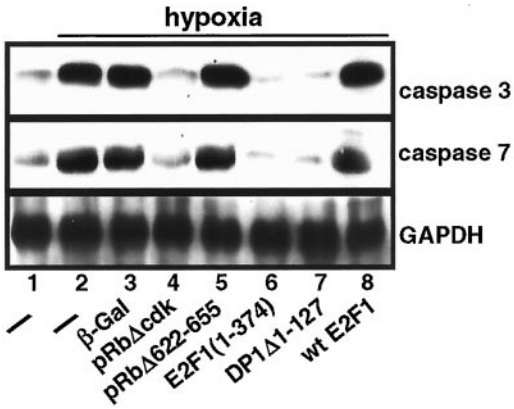

Figure 7. Induction of E2F1-dependent transcription of proapoptotic genes in cardiomyocytes during hypoxia-mediated apoptosis. Total RNA was extracted from cardiomyocytes at 20 hours after exposure to hypoxia. The blot was probed with full-length cDNAs to caspases 3 and 7, and mRNA levels were normalized using GAPDH expression as described in Materials and Methods. One representative result of 3 independent experiments is depicted.

Hypoxia led to a 2.6-fold increase in luciferase activity of E2F1Luc (wt) (Figure 6B). This result is indicative of a relief of repression of the E2F sites of E2F1Luc (wt) in apoptotic cardiomyocytes, inasmuch as (1) overexpression of E2F1(E132), E2F1(1-374), DP1Δ1-127, dn.cdk2/3, and pRbΔcdk but not pRbΔ622-655 inhibited the luciferase reporter significantly (Figure 6B). Similar results were obtained with cdc25A reporter constructs carrying wt and mt E2F sites (data not shown).[22] We conclude from these data that hypoxia also evokes a cdk2/3-dependent derepression of E2F1-regulated genes.

Hypoxia-Induced Apoptosis Requires E2F1-Dependent Gene Transcription of Proapoptotic Factors in Cardiomyocytes

To analyze whether the inhibition of apoptosis by inactivated components of the pRb pathway involves downregulation of E2F-regulated factors, Northern blot experiments were performed using full-length cDNAs to the E2F-regulated proapoptotic genes of caspase 3 and caspase 7.[23] The mRNA levels of both caspase 3 and caspase 7 were significantly increased in cardiomyocytes in the presence of DFx (Figure 7). In contrast, hypoxic treatment of cells expressing transcriptionally inert E2F1(1-374) or DP1Δ1-127 markedly reduced the transcript levels of caspases 3 and 7. Therefore, induction of caspase 3/7 gene transcription required E2F transactivation ability. This was further corroborated by ectopic pRbΔ622-655, which, in contrast to pRbΔcdk, did not antagonize the hypoxia-dependent induction of caspase 3/7 mRNA expression (Figure 7). Taken together, our data suggest that hypoxia-mediated cell death involves transcriptional transactivation of E2F-regulated proapoptotic genes.

Discussion

The present study analyzed the role of cell cycle–regulating factors controlling the pRb-dependent signaling pathway in hypoxia-induced cardiomyocyte apoptosis. Our data show that the apoptotic response of cardiomyocytes was inhibited by selective and functional inactivation of components of the retinoblastoma pathway (see the simplified diagram depicted in Figure 8).

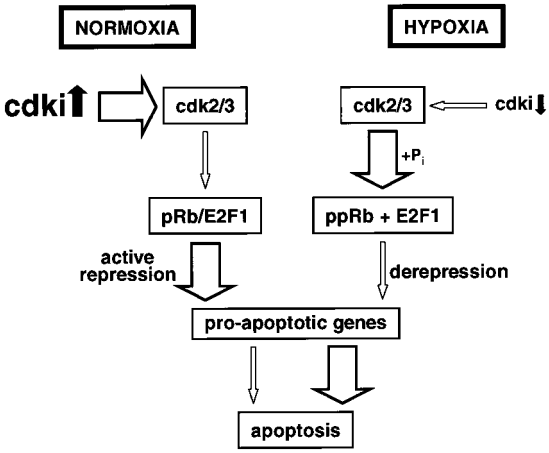

Figure 8. Modulation of retinoblastoma-dependent signaling in hypoxia-induced cardiomyocyte apoptosis. Note that this simplified schematic diagram does not include the cdk-independent antiapoptotic effects of the cdkis. pRb indicates hypophosphorylated retinoblastoma protein; ppRb, hyperphosphorylated pRb.

Protective Role of $p21^{CIP1}/p27^{KIP1}$ in Cardiomyocyte Apoptosis

Recently, cdk2 activation was shown to participate in the execution of programmed cell death in several cell types, including cardiomyocytes.[14,24,25] These studies suggested that the downregulation of the negative regulators of cdk activity, including $p21^{CIP1}/p27^{KIP1}$, is linked to the apoptosis-related activation of cdk2. Although our data support the observation of apoptosis-induced downregulation of endogenous $p21^{CIP1}/p27^{KIP1}$, they show that despite an increase in phosphotransferase activity, ectopic wt.cdk2/3 failed to reestablish hypoxia-induced cardiomyocyte apoptosis in the presence of coexpressed $p21^{CIP1}/p27^{KIP1}$ (Figure 3). We conclude from our observations that cdkis may exert their antiapoptotic effect through mechanisms unrelated to and downstream from their cdk-inhibitory potential. In this regard, recent studies in endothelial cells have demonstrated a direct interaction between $p21^{CIP1}$ and caspases, proteases that are crucial to the execution of apoptosis.[25] Also, the abrogation of monocyte apoptosis by $p21^{CIP1}$-dependent downregulation of apoptosis signal-regulating kinase 1 activity, resulting in inhibition of the stress-activated mitogen-activated protein kinase cascade, may represent a cdk-independent antiapoptotic mechanism operated by $p21^{CIP1}/p27^{KIP1}$.[26] Notably, the stress-activated mitogen-activated protein kinase cascade has been shown to be functional in cardiomyocyte apoptosis.[27,28] However, whether suppression of this signaling pathway or the direct interaction with executioner caspases is also part of the apoptosis-suppressive effect of $p21^{CIP1}/p27^{KIP1}$ in cardiomyocytes remains to be determined.

Rescue of Cardiomyocytes From Apoptosis by dn.cdk2/3

Recently, $p27^{KIP1}$ was identified as repressing apoptosis in fibroblasts induced by growth factor deprivation.[29] In these

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cells, modulation of cell death was due to $p27^{KIP1}$-dependent inhibition of cdk2. Others have shown that forced expression of cyclin A is sufficient to induce apoptosis in cardiomyocytes and that expression of a catalytically inactive mt of cdk2 was able to abrogate hypoxia-induced apoptosis.[14] Based on the notion that binding to cyclin A is required for the kinase activity of cdk2, these data point toward a crucial role of this cell cycle activator in cardiomyocyte apoptosis. Although this observation is supported by our data, we provide evidence that in cardiomyocyte apoptosis, the endogenous expression and/or activity of cdks 2, 3, 4, and 6 is regulated in a differential manner. A specific subset of cdks, namely, cdk4 and cdk6, is downregulated in expression and kinase activity, whereas protein levels were maintained, and kinase activity was increased for cdk2/3 (Figure 2). Moreover, although the specific inhibition of either cdk2 or cdk3 was sufficient to block hypoxia-induced apoptosis, our coexpression analysis revealed that these kinases could not compensate for one another (Figure 4). This finding was unexpected inasmuch as both cdks share a high degree of similarity in their amino acid composition (76% identity). Because wt.cdk2 could not overcome the antiapoptotic impact of dn.cdk3 and vice versa, it is suggested that both cdks act on separate pathways upstream from pRb. Although no endogenous cyclin-cdk3 complexes have been identified, recombinant cdk3/cyclin A was able to phosphorylate pRb in vitro and in living cells.[30] Our data suggest that cardiomyocyte apoptosis relies on the activation of cdk3 in addition to cdk2. Therefore, it appears reasonable to assume that cardiomyocyte apoptosis induced by forced expression of cyclin A is based on the activation of both cdk2 and cdk3.[14] cdk2/3 acts independently during the G_1- to S-phase transition in various mammalian cells.[8,31] As opposed to the targeting of E2F by cdk2, cdk3 binds specifically to the DP1 subunit of E2F/DP heterodimers. Therefore, cdk2/3 directly participates in the regulation of the G_1- to S-phase transition. Because SV40 large-T antigen induced a release of E2F from pocket proteins in the presence of dn.cdk2 but not dn.cdk3, both kinases might operate different cellular pathways upstream from pRb.[8,31] Our observations not only describe a functional role of cdk2/3 in apoptosis, but, to the best of our knowledge, they are also the first to demonstrate a unique recruitment of cell cycle factors in cardiomyocyte apoptosis compared with other cell types. For example, neuronal cells and cardiomyocytes, albeit both being terminally differentiated, seem to use a distinct pattern of cell cycle factors in this context. In neurons, nerve growth factor withdrawal or ischemia-induced apoptosis was blocked by overexpression of dn.cdk4/6 but not dn.cdk2/3, at least partially explaining why in these cells ectopic $p16^{INK4}$ (besides $p21^{CIP1}$/$p27^{KIP1}$) could act as a potent inhibitor of cell death.[32]

Regulation of Apoptosis by pRb/E2F in Cardiomyocytes

The present study delivers sustained experimental evidence indicating that pRb-mediated protection from hypoxia-induced apoptosis in cardiomyocytes is mediated through neutralization of E2F transactivation potential. E2F selectively binds to hypophosphorylated pRb, and functional inactivation of pRb requires its sequential modification by G_1 cdk activities.[7] Both transactivation and transderepression of E2F-dependent promoters occur during cardiomyocyte cell death, suggesting that transrepression of E2F-controlled genes is required for their survival (Figure 5). This view is further substantiated by the effect of transactivating-deficient E2F1/DP1 mts, which efficiently protect cardiomyocytes from hypoxia-evoked cell death. On the basis of these results, hypoxia-triggered apoptosis appears to require the elimination of growth-suppressive pRb, and interventions compromising the impact of physiologically inactivated pRb blocked cell death in cardiomyocytes (Figure 4). This is surprising in light of the function of pRb as a tumor suppressor, which actually is expected to promote cell death (like p53) instead of functioning as an apoptosis protection factor.[6] However, pRb is able to function as an essential determinant for the inhibition of apoptosis, inasmuch as it has been demonstrated for neuronal and skeletal muscle cell death and in a subset of cancer cell lines.[33–35] Importantly, pRb-deficient muscle and neuronal cells display a markedly increased apoptosis rate, which is significantly decreased in pRb-deficient/E2F1-deficient double mts.[36] These data suggest that pRb-mediated protection of cell death proceeds through the binding and inactivation of E2F1. Overexpression of E2F1 and not E2F2/3 reportedly leads to apoptosis.[37] Phillips et al[38] have shown that E2F1 induces apoptosis in Saos-2 cells by downregulation of TRAF2 expression, which prevents the activation of the antiapoptotic factors nuclear factor-κB and c-Jun N-terminal kinase. Taken together, the control of E2F1 function probably is the prevalent mechanism of pRb-dependent protection from apoptosis. Interestingly, our group found that the ETS-related transcription factor GABPγ1 suppresses E2F1-dependent apoptosis by mechanisms unrelated to the inhibition of the transactivation capacity of E2F1, providing a pRb-independent mechanism for regulating E2F-dependent apoptosis. Therefore, future studies aiming at the molecular dissection of the relative contribution of pRb family members during the regulation of cell death in differentiated cardiomyocytes are warranted.

Acknowledgments

This study was supported in part by the Deutsche Forschungsgemeinschaft (DFG, Ha 1777/7-1) and a grant-in-aid from the Max-Delbrück-Center to Dr von Harsdorf. We thank Daniela Grothe for the construction of recombinant adenoviral constructs.

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