p21 Blocks Irradiation-Induced Apoptosis Downstream of Mitochondria by Inhibition of Cyclin-Dependent Kinase–Mediated Caspase-9 Activation

Dennis Sohn, Frank Essmann, Klaus Schulze-Osthoff, and Reiner U. Jänicke

Institute of Molecular Medicine, University of Düsseldorf, Düsseldorf, Germany

Abstract

The role of the cyclin-dependent kinase (CDK) inhibitor p21 as a mediator of p53-induced growth arrest is well established. In addition, recent data provide strong evidence for new emerging functions of p21, including a role as a modulator of apoptosis. The mechanisms, however, by which p21 interferes with the death machinery, especially following ionizing radiation (IR), are largely unknown. Here, we report that IR induced caspase-9 and caspase-3 activation and subsequent apoptosis only in p21-deficient colon carcinoma cells, whereas similar treated wild-type cells were permanently arrested in the G_2-M phase, correlating with the induction of cellular senescence. Interestingly, activation of the mitochondrial pathway, including caspase-2 processing, depolarization of the outer mitochondrial membrane, and cytochrome *c* release, was achieved by IR in both cell lines, indicating that p21 inhibits an event downstream of mitochondria but preceding caspase-9 activation. IR-induced p21 protein expression was restricted to the nucleus, and no evidence for a mitochondrial or cytoplasmic association was found. In addition, p21 did neither interact with caspase-3 or caspase-9, suggesting that these events are not required for the observed protection. Consistent with this assumption, we found that CDK inhibitors potently abrogated IR-induced caspase processing and activation without affecting mitochondrial events. In addition, *in vitro* caspase activation assays yielded higher caspase-3 activities in extracts of irradiated p21-deficient cells compared with extracts of similar treated wild-type cells. Thus, our results strongly indicate that p21 protects cells from IR-induced apoptosis by suppression of CDK activity that seems to be required for activation of the caspase cascade downstream of the mitochondria. (Cancer Res 2006; 66(23): 11254-62)

Introduction

When proliferating cells encounter a genotoxic stress induced for instance by ionizing radiation (IR) or chemotherapeutic drugs, the cell cycle must be arrested immediately to ensure DNA integrity. This event is usually followed by the decision of whether the cells remain arrested in the cell cycle, initiate DNA repair, or execute the apoptotic program. One of the main players involved in this process is the tumor suppressor protein p53 that is known to accomplish these tasks via transcription-dependent and transcription-independent events (1). Whereas cell cycle arrest induced by p53 is mediated by only a few proteins, including the cyclin-dependent kinase (CDK) inhibitor p21, numerous candidates are known to be involved in p53-dependent apoptosis. Among them, the proapoptotic members of the Bcl-2 family (Bax, Noxa, and Puma) are certainly the most important players (2). They instigate the intrinsic or mitochondrial death pathway either directly via disruption of the outer mitochondrial membrane or indirectly by sequestering antiapoptotic proteins, such as Bcl-2 and Bcl-x_L (3). Recently, it was also shown that p53 itself translocates to mitochondria during genotoxic stress where it activates this pathway via binding to these antiapoptotic proteins (4). Although such a mechanism could nicely explain its transcription-independent proapoptotic activity, p53 was also found associated with mitochondria in the absence of apoptosis (5). Nevertheless, disruption of the outer mitochondrial membrane results in the release of cytochrome *c* that together with apoptosis-activating factor 1 (APAF-1) and caspase-9 forms a high molecular weight complex, the apoptosome. Oligomerization of caspase-9 in the apoptosome then leads to its autocatalytic processing and subsequent activation of caspase-3 that is known to cleave the majority of cellular substrates during apoptosis (6).

Thus, p53-dependent apoptosis is well documented and appreciated as a valuable facet of its tumor suppressive activities. Intriguingly, p53 was also shown to possess antiapoptotic capabilities, as several cells lacking functional p53 are under certain circumstances even more sensitive to apoptosis than their p53-proficient counterparts (7). With regard to this, we have also shown recently that IR induced a decrease in the mitochondrial membrane potential only in MCF-7 breast carcinoma cells stably expressing a p53 small interfering RNA, suggesting that the presence of a wild-type p53 contributes to the radioresistant phenotype of these cells (8). Although the mechanisms responsible for the antiapoptotic effect of p53 are far from being elucidated, accumulating evidence suggests the involvement of p21 in this process. Inhibition of DNA damage–induced p21 expression for instance rendered MCF-7 cells sensitive toward IR-induced apoptosis in a caspase-3-dependent manner, whereas overexpression of p21 reverted this effect (9). In addition, wild-type HCT116 cells were efficiently chemosensitized and radiosensitized by either homologous disruption of the *p21* gene or by p21 antisense oligonucleotides both *in vitro* and in tumor xenografts in nude mice (10, 11). Moreover, whereas p21-proficient tumors underwent regrowth following treatment with γ-radiation, a significant fraction of p21-deficient tumors were completely eradicated (12). Although p21 was shown in the following years to confer resistance to a variety of apoptotic stimuli, little is known about the underlying mechanisms (13, 14).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

D. Sohn and F. Essmann contributed equally to this work.

Requests for reprints: Reiner U. Jänicke, Institute of Molecular Medicine, University of Düsseldorf, Building 23.12, Universitätsstrasse 1, D-40225 Düsseldorf, Germany. Phone: 49-211-8115973; Fax: 49-211-8115892; E-mail: janicke@uni-duesseldorf.de.

doi:10.1158/0008-5472.CAN-06-1569

Many diverse signaling pathways have been proposed to be involved in the p21-mediated protection from apoptosis, including inhibition of the apoptosis signal-regulating kinase 1 (ASK1) and c-Jun NH_2-terminal kinase (JNK), and even the direct binding and inhibition of caspase-3 was described (15–17). Interestingly, it was shown that phosphorylation of p21 by the Akt kinase not only leads to its stabilization but also to a redistribution of p21 into the cytoplasm, thereby contributing to the prosurvival activities of both Akt and p21 (18–20). In other studies, cytoplasmic localization of p21 was forced by removal of the two nuclear localization sequences at the COOH terminus either by the caspase-3-mediated cleavage at Asp^{112} or following deletion of its COOH-terminal amino acids (21, 22). These truncations abrogated the survival function of p21 and correlated with increased CDK activities. Elevated CDK activities were also observed in several other apoptosis systems, and inhibition of CDK activity prevented several manifestations of apoptotic death, including chromatin condensation (23, 24). Thus, these data provided a causal link between the activity of these cell cycle regulatory kinases and the execution of the cell death program. With occasional exceptions (25), however, CDKs became only activated in a caspase-dependent manner, and activation of caspases was unimpaired in cells in which CDK activity was inhibited. In contrast, CDK inhibition was also shown to induce caspase activation and cell death, a phenomenon that is presently explored in phase II clinical trials (26). Based especially on the lack of information on possible CDK targets, it is still very much unresolved whether these cell cycle regulatory kinases are indeed required for induction of apoptosis, and if so, at which step they interact with the apoptotic machinery.

To further delineate the mechanisms by which p21 confers resistance, we compared apoptosis susceptibilities of checkpoint-deficient ($p53^{-/-}$ and $p21^{-/-}$) HCT116 cells with their wild-type counterparts toward IR treatment. We found the p21 protein in IR-resistant HCT116 wild-type cells almost exclusively localized in the nucleus, and no evidence was detected for an association of p21 with caspase-3 or caspase-9 that would explain its protective role. Interestingly, although IR induced processing of caspase-2 and activation of the mitochondria and cytochrome *c* release in all three cell lines, including the wild-type cells, the post-mitochondrial caspase-9 and caspase-3 were only activated in p21- and p53-deficient cells. Together with our observation that the specific CDK inhibitor roscovitine blocked IR-induced post-mitochondrial caspase activation without affecting the loss of the mitochondrial membrane potential, our data suggest that p21 protects cells via inhibition of CDKs that are required downstream of the mitochondria for an efficient activation of the caspase cascade.

Materials and Methods

Cell lines, reagents, and antibodies. HCT116 wild-type cells and their checkpoint-deficient variants were maintained in McCoy's 5A medium supplemented with 10% heat-inactivated FCS, 10 mmol/L glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin (PAA Laboratories, Linz, Austria). The pan-caspase inhibitory peptide Q-VD-OPH (Q-Val-Asp-CH_2-O-Ph) and the caspase-2 and caspase-3 inhibitory peptides z-VDVAD-fmk (z-Val-Asp-Val-Ala-Asp-fluoromethylketone) and z-DEVD-fmk (z-Asp-Glu-Val-Asp-fluoromethylketone) were from MP Biomedicals (Irvine, CA). The fluorogenic caspase-3 and caspase-9 substrates DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin) and LEHD-AMC (*N*-acetyl-Leu-Glu-His-Asp-aminomethylcoumarin) were from Biomol (Hamburg, Germany). The polyclonal antibodies against caspase-3 and caspase-9 were from R&D Systems (Wiesbaden, Germany) and from Cell Signaling Technology (Danvers, MA), respectively. The rat caspase-2 monoclonal antibody (mAb) as well as the CDK inhibitors roscovitine and olomoucine were from Alexis Biochemicals (Lausen, Switzerland). The p53 mAb (Ab-6) was from Calbiochem (Bad Soden, Germany), whereas the mAbs recognizing cytochrome *c*, TOM20, poly(ADP-ribose) polymerase (PARP), and p21 and the polyclonal antibodies directed against the high mobility group 1 (HMG1) protein were from BD Biosciences (Heidelberg, Germany). The mAbs towards Bcl-2 and Bax were from Novocastra Laboratories (Newcastle, United Kingdom) and Trevigen (Gaithersburg, MD), respectively. The mAb recognizing the proliferating cell nuclear antigen and the polyclonal antibody to CDK2 was from Santa Cruz (Heidelberg, Germany). The actin mAb, the nuclear stain 4′,6-diamidino-2-phenylindole, and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin were from Sigma (Deisenhofen, Germany). Peroxidase-labeled secondary antibodies were from Promega GmbH (Mannheim, Germany), and the secondary chicken anti-mouse antibody coupled to Alexa Fluor 488 that was used for the immunofluorescence studies were from Molecular Probes (Mobitech, Göttingen, Germany).

Treatment of cells and measurement of cell death. Cells were exposed to IR (usually 20 Gy) using a Gammacell 1000 Elite (Nordion International, Inc., Fleurus, Belgium). Cell death was assessed by microscopic examination or by determination of the lactate dehydrogenase (LDH) activity in supernatants of 10^5 cells according to the protocol of the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The values obtained are given in arbitrary units. These supernatants were also analyzed on SDS-polyacrylamide gels for the death-associated release of HMG1.

Senescence-associated β-galactosidase staining and immunofluorescence microscopy. Staining for β-galactosidase activity was done as described (8). Pictures were taken on an Axiovert135 microscope (Zeiss, Jena, Germany) with an Apochromat ×20 objective using OpenLab software (Improvision, Tübingen, Germany). Immunofluorescence staining was done as described (5). Pictures were taken using a Zeiss LSM 510 Meta equipped with a ×40 oil immersion objective.

Preparation of cell extracts, subcellular fractionation, and Western blotting. Total cell extracts were prepared in high-salt lysis buffer containing 1% NP40, 20 mmol/L HEPES, 2 mmol/L PMSF, 350 mmol/L NaCl, 20% glycerol, 1 mmol/L $MgCl_2$, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 0.5 mmol/L DTT, and protease inhibitors. The preparation of highly purified mitochondrial M2 fractions by a discontinuous 1.2 to 1.6 mol/L sucrose gradient was done as described (5). Protein concentrations were determined with the Bio-Rad protein assay. Subsequently, proteins were separated on SDS-polyacrylamide gels and electroblotted to polyvinylidene difluoride membranes (Amersham, Braunschweig, Germany). Following antibody incubation, the proteins were visualized by enhanced chemiluminescent staining using enhanced chemiluminescence reagents (Amersham Biosciences, Freiburg, Germany).

Determination of the mitochondrial transmembrane potential and cytochrome *c* release by fluorescence-activated cell sorting analyses. The mitochondrial transmembrane potential ($\Delta\Psi_m$) was analyzed by flow cytometry using the $\Delta\Psi_m$-specific stain TMRE (Molecular Probes) as described (8). For the measurement of mitochondrial cytochrome *c* release (27), 10^5 cells were suspended in 250 μL permeabilization buffer (50 μg/mL digitonin, 2 mmol/L EDTA, 100 mmol/L KCl) in PBS and fixed for 20 minutes at room temperature by addition of 300 μL of 8% formaldehyde followed by three PBS washes. Cells were incubated for 1 hour in 300 μL blocking buffer (3% bovine serum albumin, 0.05% saponin in PBS) at 4°C followed by the addition of 1.5 μL of the cytochrome *c* antibody and overnight incubation at 4°C. Samples were washed thrice, and secondary phycoerythrin-conjugated goat anti-mouse antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 hour at room temperature in the dark. Cells were washed and resuspended in 200 μL PBS, and cytochrome *c* staining was determined by flow cytometric analysis on a FACSCalibur (Becton Dickinson, Heidelberg, Germany) using the FL2-histogram profile and the CellQuest software.

Immunoprecipitation. Cell extracts were prepared from 1×10^7 cells in 500 μL lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40] containing protease inhibitors. For the precipitation, the protein

concentrations were adjusted, and 2 μg antibody and 30 μL protein G-Sepharose (Sigma) were added to the extracts and rotated overnight at 4°C. The Sepharose beads were washed in lysis buffer and analyzed by SDS-PAGE and Western blotting.

***In vitro* caspase activation assay and fluorometric determination of caspase activity.** For *in vitro* activation of caspases, cells were resuspended in low salt buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 2 mmol/L $MgCl_2$, 1 mmol/L EDTA] containing protease inhibitors and 1 mmol/L DTT. After incubation for 15 minutes at 4°C, cells were disrupted by 30 passages through a 20-gauge needle. Cell extracts were cleared twice by centrifugation (10,000 × *g*) at 4°C for 15 minutes each and adjusted to an equal protein concentration. *In vitro* activation reactions were started by adding 10 mmol/L DTT, 2 mmol/L dATP, 1 mmol/L $MgCl_2$ in the presence of 3.5 μmol/L cytochrome *c* (from horse heart; Sigma) to 200 μg aliquots in 75 μL and incubation at 37°C. Caspase-3 and caspase-9 activities were assessed after 60 minutes using 50 μg aliquots of each reaction in the fluorometric DEVD and LEHD cleavage assays as described (28). Student's *t* test was employed for statistical analysis.

Results

IR induces apoptosis only in checkpoint-deficient HCT116 cells. To delineate the underlying mechanisms by which the CDK inhibitor p21 exerts its antiapoptotic effect, we first exposed wild-type HCT116 cells and their checkpoint-deficient ($p53^{-/-}$ and $p21^{-/-}$) counterparts to a single dose of 20 Gy γ-irradiation and assessed cell death induction by several means. Remarkably, during a period of 7 days after IR, that represents time points not considered in previous studies, only marginal signs of cell death were detected in wild-type cells. These cells did not release a substantial amount of either the enzyme LDH or the HMG1 protein into their supernatants (Fig. 1*A* and *B*). Instead, they entered a permanent cell cycle arrest in the G_2-M phase that correlated with the induction of cellular senescence as measured by staining of the senescence-associated β-galactosidase activity (Fig. 1*C*). In contrast, IR treatment clearly induced death of the two checkpoint-deficient HCT116 cell lines as evidenced by a significant time-dependent release of LDH and HMG1 into their supernatants (Fig. 1*A* and *B*) in the absence of any detectable β-galactosidase activity (Fig. 1*C*; data not shown). Hence, expression of p53 and p21 (see Fig. 5) critically influences the outcome of an IR treatment.

The IR-induced cell death was most likely mediated via apoptosis because the death-associated release of LDH and HMG1 could be efficiently blocked by the pan-caspase inhibitory

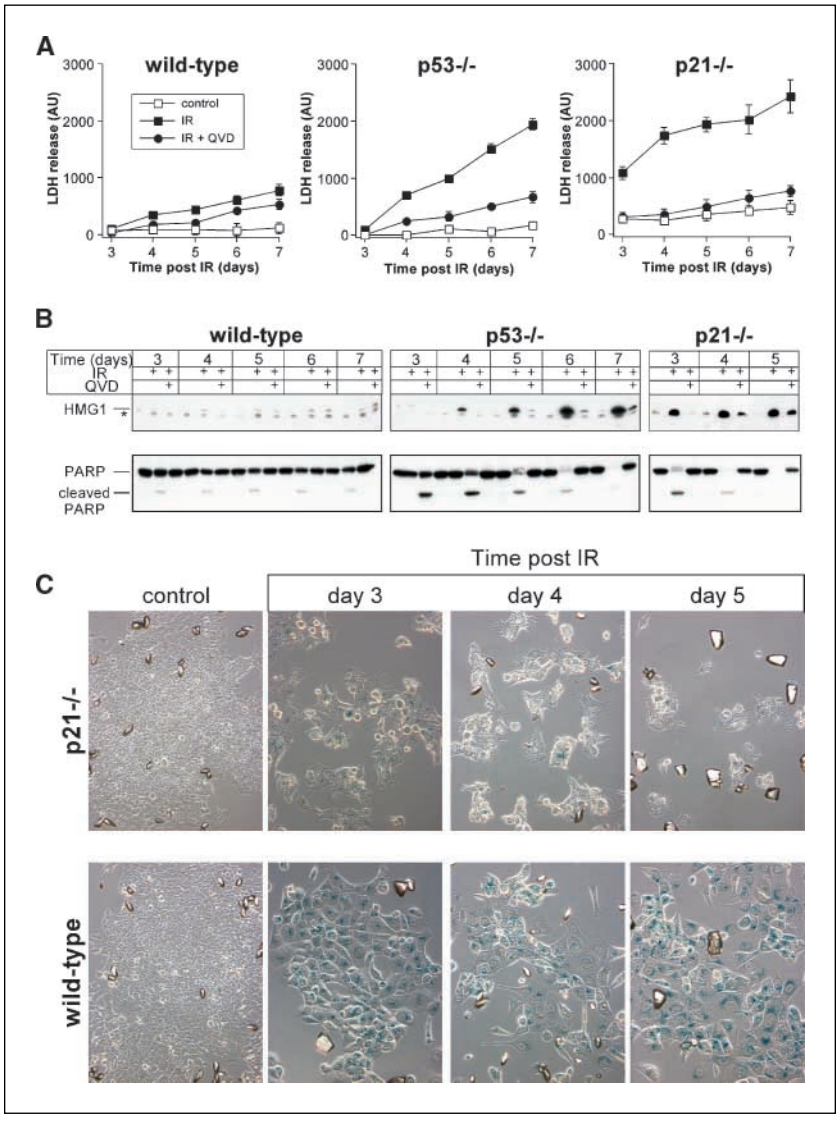

Figure 1. IR induces apoptosis of checkpoint-deficient HCT116 cells but not of their wild-type counterpart. Wild-type and checkpoint-deficient cells were either left untreated or were exposed to IR in the absence or presence of the pan-caspase inhibitory peptide QVD (10 μmol/L). After the indicated days, cell death was determined by the release of LDH (*A*) and HMG1 (*B*) into the supernatant, or by cleavage of PARP that represents a typical caspase substrate. QVD was added at days 0, 3, and 5. *Points,* mean of three independent experiments; *bars,* SD. *, band of unknown origin. *C,* IR treatment results in the p21-dependent induction of cellular senescence. Wild-type and p21-deficient cells were either left untreated or were exposed to IR and analyzed after the indicated days for the induction of cellular senescence by staining of the senescence-associated β-galactosidase activity. One representative experiment out of two.

peptide Q-VD-OPH (QVD; Fig. 1). In addition, cleavage of PARP that was predominantly observed in irradiated p21- and p53-deficient cells was inhibitable by QVD, implying that caspases were responsible for the observed cell deaths (Fig. 1*B, bottom*). Indeed, IR induced the processing of caspase-9 and caspase-3 in both checkpoint-deficient cell lines, as indicated by the decrease of the procaspase-9 protein and the appearance of the active caspase-3 subunits (Fig. 2*A*). In addition, activation of both caspases was confirmed by using the fluorogenic DEVD and LEHD substrate cleavage assays that measure caspase-3 and caspase-9 enzymatic activities, respectively (Fig. 2*B*). Interestingly, although neither caspase-3 nor caspase-9 were found to be processed or activated in IR-treated HCT116 wild-type cells, confirming their resistance to this treatment, procaspase-2 was processed in all three cell lines to a similar extent (Fig. 2*A, top*). Thus, our results show that only the two checkpoint-deficient cell lines undergo apoptosis following exposure to IR and suggest that caspase-2 is unable to directly activate caspase-9 and caspase-3. More importantly, as caspase-2 is considered to act upstream of the mitochondria in many apoptotic systems (29), our results indicate that the apoptosis resistance of wild-type cells toward IR might be caused by a p21-mediated event located at or downstream of the mitochondria.

Expression of p21 does not interfere with the IR-induced activation of mitochondria. To characterize such an event, we first asked at which step p21 interacts with the apoptotic cascade. Therefore, we exposed wild-type and p21-deficient cells to IR and analyzed their mitochondrial membrane potential ($\Delta\Psi_m$). Interestingly, loss of $\Delta\Psi_m$, an event that is closely associated with the onset of apoptosis (30), was not only observed in p21-deficient cells

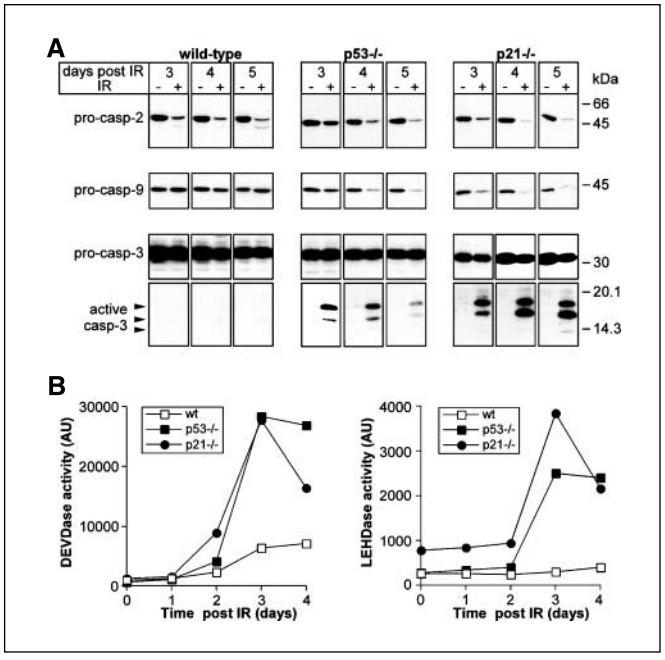

Figure 2. Caspase processing and activation in irradiated HCT116 cell lines. *A,* extracts of cells that were either left untreated or exposed to IR were analyzed by Western blotting after the indicated days for the processing of the indicated caspases. The arrows indicate active caspase-3 subunits. *B,* extracts of cells that were either left untreated or exposed to IR were analyzed after the indicated days for caspase-3 (DEVDase) and caspase-9 (LEHDase) enzymatic activities. Note that caspase-9 and caspase-3 were only processed and activated in checkpoint-deficient cells, whereas caspase-2 was processed in all three cell lines. One representative experiment out of three.

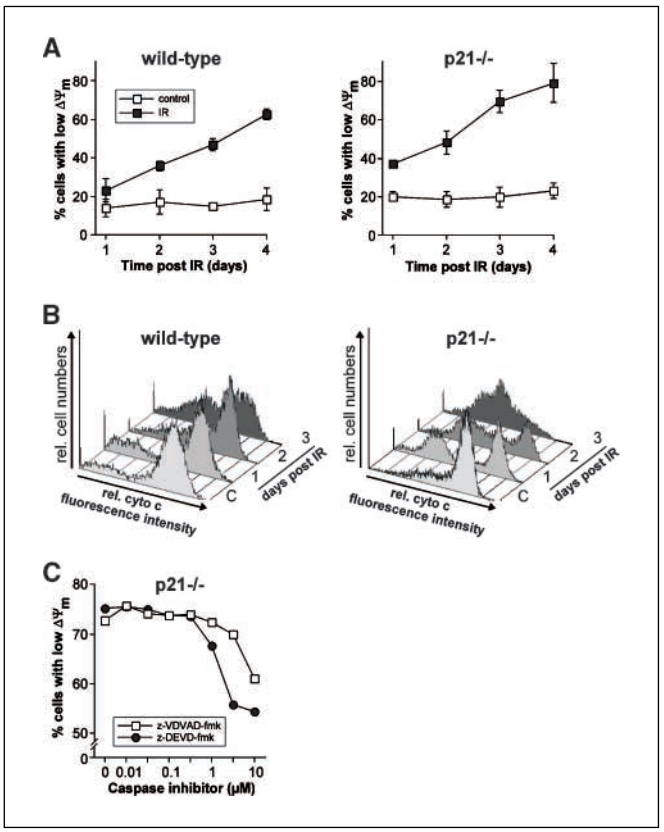

Figure 3. IR activates also the mitochondria in apoptosis-resistant HCT116 wild-type cells. *A,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of untreated (*control*) and irradiated (*IR*) cells after the indicated times. *Points,* mean of three independent experiments; *bars,* SD. *B,* measurement of cytochrome c release from the mitochondria of untreated (*C*) and irradiated cells after the indicated times. One representative experiment out of three. *C,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of p21-deficient cells 3 days after irradiation in the absence or presence of the indicated caspase inhibitory peptides. One representative experiment out of two.

but also to an almost similar extent in their IR-resistant wild-type counterparts (Fig. 3*A*). Furthermore, mitochondrial cytochrome *c* release occurred in a time-dependent manner also in wild-type cells, although not as efficiently as observed in p21-deficient cells (Fig. 3*B*). This is most likely due to the fact that activation of mitochondria is, at least partially, amplified in a caspase-dependent manner (31), an event unlikely to be achieved in wild-type cells due to the failure of IR to activate caspase-9 and caspase-3. Consistent with this, we found indeed that the IR-induced loss of $\Delta\Psi_m$ in p21-deficient cells could be partially rescued by the caspase inhibitory peptides VDVAD-fmk and DEVD-fmk that block caspase-2 and caspase-3, respectively (Fig. 3*C*). Thus, together with our previous data showing only activation of caspase-2, but not of caspase-9 or caspase-3 in irradiated HCT116 wild-type cells, these results clearly narrow down the interference of p21 with the mitochondrial death pathway to an event that follows cytochrome *c* release but precedes activation of caspase-9.

IR-induced p21 protein does not bind caspase-3 and caspase-9 and is not associated with mitochondria or localized in the cytoplasm. Previously, p21 was reported to bind to procaspase-3, thereby preventing its activation (17). The same group further postulated that mitochondria are essential for the procaspase-3/p21 complex formation (32). Intriguingly, this would

perfectly match the series of events in our aforementioned hypothesis, and the obvious lack of such an event in p21-deficient cells might indeed contribute to their increased sensitivity. Therefore, we analyzed whether p21 is associated with caspase-3 in irradiated HCT116 wild-type cells. We immunoprecipitated caspase-3 from extracts of untreated cells and from cells 1 day after IR under conditions that allow coprecipitation of associated proteins, as we have successfully shown previously (33). However, although caspase-3 was almost completely precipitated from extracts of control and irradiated cells, p21 was not detected in these precipitates (Fig. 4*A*). In addition, the reverse approach (i.e., the immunoprecipitation of p21) did not yield in the coprecipitation of caspase-3 or caspase-9 (Fig. 4*B*). The faint caspase-3 signal detected in the precipitates of irradiated cells does not represent caspase-3 specifically coprecipitated with p21, as it was also present in control reactions that were done in the absence of the p21 antibody (data not shown). The p21 interacting target CDK2 in contrast, was successfully coprecipitated under these conditions, thus validating our approach. Similar results were obtained when immunoprecipitations were done with extracts of cells prepared 2 days after IR (data not shown). Thus, these data argue against a direct association of p21 with caspases in HCT116 wild-type cells and, hence, do not explain the antiapoptotic activity of p21.

As mitochondria were postulated to be essential for the procaspase-3/p21 complex formation (32), we also examined a possible association of p21 with these organelles. However, p21 expression induced by either IR or the chemotherapeutic drug camptothecin was exclusively found in nuclei-containing total cellular extracts but not in highly purified mitochondrial M2 fractions (Fig. 4*C*) that were previously shown to contain p53 protein (5). In addition, and consistent with these results, immunofluorescence studies also showed that p21 expression was almost exclusively confined to the nuclei of irradiated HCT116 wild-type cells that displayed a dramatic increase in size due to this treatment (Fig. 4*D*).

Analysis of apoptosis-relevant proteins in wild-type and checkpoint-deficient HCT116 cells. Next, we compared p53, Bax, and Bcl-2 expression in these cell lines, as it was postulated that the sensitivity of p21-deficient HCT116 cells to drug-induced apoptosis is caused by an increased expression of p53 and the subsequent modification of the Bax/Bcl-2 ratio (34). We found indeed higher protein levels of p53 and Bax in p21-deficient cells when compared with their wild-type counterparts (Fig. 5). Consistent with higher p53 expression, Bcl-2 levels were also found to be reproducibly lower in p21-deficient cells, as this antiapoptotic protein is transcriptional repressed by p53 (Fig. 5; ref. 35). However, we noticed that $p53^{-/-}$ cells that also lack detectable levels of p21 do undergo IR-induced apoptosis (Figs. 1 and 2), although they display significant lower Bax and higher Bcl-2 protein expression when compared with wild-type cells (Fig. 5). Thus, besides a hyperfunctional p53 (34, 36), additional mechanisms must be involved in the increased IR sensitivity of p21-deficient cells.

CDK inhibitor roscovitine blocks IR-induced caspase activation but has no influence on the mitochondrial membrane potential. As we could neither verify a cytoplasmic localization of p21 nor its binding to caspase-3 or caspase-9, which would have argued for a direct effect of p21, we hypothesized that its protective

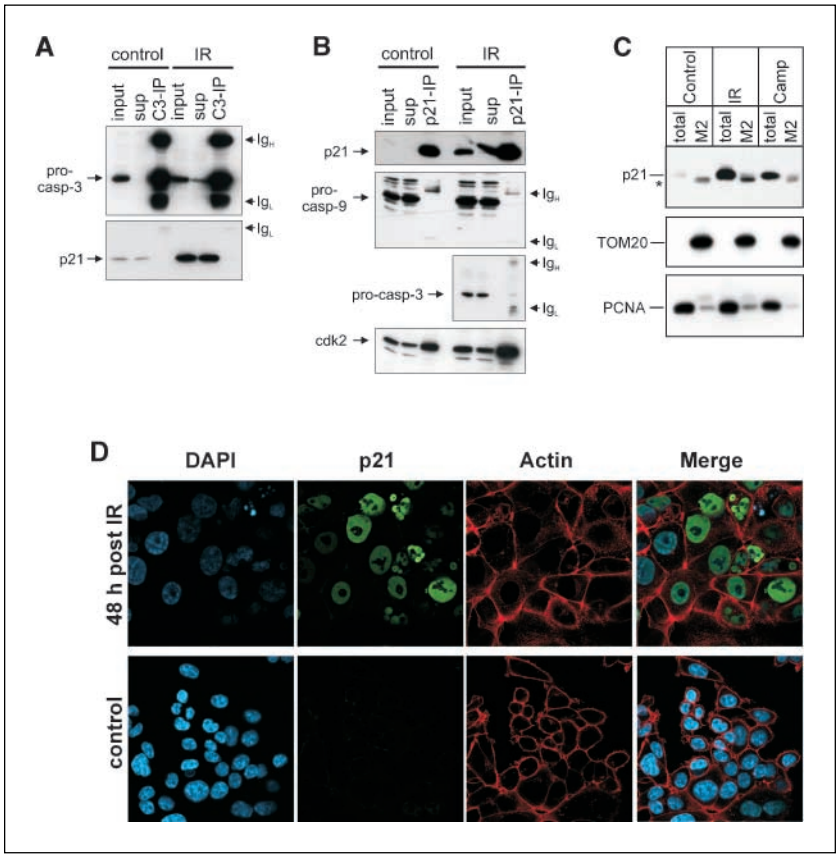

Figure 4. p21 does not associate with caspase-3 or caspase-9. Extracts of wild-type cells that were either left untreated or exposed to IR were subjected to Western blot analysis either before (*input*) or after (*sup*) immunoprecipitation with the caspase-3 antibody (*A*) or the p21 antibody (*B*). The immunoprecipitates for caspase-3 and p21 are labeled as C3-IP and p21-IP, respectively. As a control for the p21 immunoprecipitation, the presence of cdk2 was confirmed by Western blot. One representative experiment out of two. *C* and *D,* p21 is localized in the nucleus but not in the cytoplasm or mitochondria. *C,* wild-type cells were either left untreated or exposed to IR or 1 μmol/L camptothecin (*camp*). After 48 hours, total cell extracts or highly purified mitochondrial fractions (M2) were prepared and analyzed by Western blotting for the presence of p21 (*top*). *, band of unknown origin. The composition and purity of the fractions were confirmed by probing the membrane with antibodies recognizing Tom20 and PCNA that represent mitochondrial and nuclear markers, respectively. *D,* immunofluorescence staining of p21 in untreated wild-type cells (control) or in cells 48 hours following IR. Cells were also stained with the nuclear dye 4′,6-diamidino-2-phenylindole (*DAPI*) and phalloidin-TRITC that labels actin. Note the almost exclusive nuclear localization of p21 and the dramatic increase in size of irradiated cells.

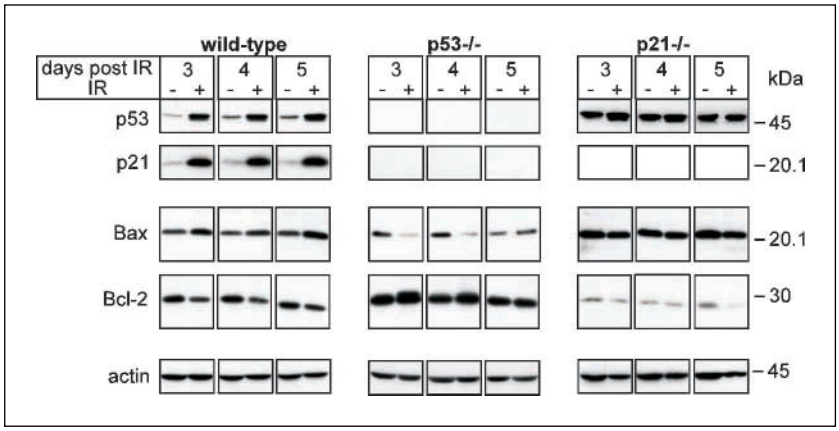

Figure 5. No correlation between p53, Bax, and Bcl-2 expression and IR-induced apoptosis. Extracts of cells that were either left untreated or exposed to IR were analyzed by Western blotting after the indicated days for the status of the indicated proteins. One representative experiment out of two.

function is due to a nuclear event, most likely mediated indirectly via inhibition of CDKs. We first attempted to confirm this by using a cellular system described by Cayrol et al. (37), in which DLD1 carcinoma cells were engineered to stably express either p21 wild-type protein or a p21 mutant deficient for CDK interaction (p21-CDK$^-$), both of which were placed under the control of a tetracycline-regulated promoter (Tet off). Only the expression of p21 wild-type protein partially rescued the cells from IR-induced apoptosis, whereas DLD1 cells expressing the p21-CDK$^-$ mutant protein were as sensitive to this treatment as uninduced (Tet on) p21-deficient control cells (Supplementary Fig. S1). Although these results are in agreement with our hypothesis, the observed effect was not as pronounced as in the HCT116 cell system. Western blot analysis revealed that this was most likely due to the down-regulation of both p21 proteins in DLD1 cells following IR (Supplementary Fig. S1), which is in sharp contrast to the results obtained with the HCT116 wild-type cells (Fig. 5).

To further explore the role of CDKs in irradiation-induced apoptosis, we exposed p21-deficient HCT cells to IR in the presence of roscovitine that is one of the most specific pharmacologic CDK inhibitors (38). Roscovitine efficiently prevented IR-induced caspase-9 and caspase-3 activation in a time- and dose-dependent manner, as shown by the absence of the active caspase subunits in the presence of this inhibitor (Fig. 6*A* and *B*). Inhibition of caspase-3 activity by roscovitine was further confirmed using the DEVD substrate cleavage assay (Fig. 6*C*). Similar results were obtained when the experiments were done in the presence of the CDK inhibitor olomoucine (data not shown), indicating that activated CDKs are essential for IR-induced apoptosis. Interestingly, and in agreement with our hypothesis that p21 functions downstream of mitochondria, caspase inhibition by roscovitine was achieved without affecting the IR-induced loss of $\Delta\Psi_m$ (Fig. 6*D*). This was further supported by our finding that activation of the pre-mitochondrial acting caspase-2 was also not affected by roscovitine (Fig. 6*A*). Together, these data provide strong evidence that suppression of CDK activity represents an event that is critically involved in the protective function of p21. In addition, the results suggest that CDKs interfere with the death pathway downstream of the mitochondria but upstream of caspase-9 activation.

Therefore, we speculated that active CDKs must positively interfere with apoptosome formation. As a reasonable approach to examine such a scenario, we did gel filtration analyses to compare apoptosome formation in irradiated p21-deficient and wild-type HCT116 cells. However, all our attempts using this approach failed, as we were unable to detect an active apoptosome complex. This is most likely due to the inhomogeneity of the irradiated cells that at any given time point after IR form an active apoptosome only in a fraction of the cells, which seems to be too little for a successful visualization by gel filtration and subsequent Western blot analyses.

Therefore, we used an alternative procedure (i.e., an *in vitro* caspase activation assay) that reflects, at least to a certain extent, their capabilities to form an apoptosome. For this purpose, we harvested the cells 24 hours after IR, as this represents a time point at which all extracts showed comparable caspase-3 activities in the absence of cytochrome *c* (no activation; Fig. 6*E*). In the presence of cytochrome *c* (activation), however, we found a significantly higher caspase-3 activity in extracts of irradiated compared with untreated p21-deficient cells. This difference was not observed between extracts of untreated and irradiated p21-proficient wild-type cells. Together, these data show that the intrinsic death pathway, although efficiently instigated by IR, requires additional signals that are only provided in the absence of p21, most likely by active CDKs, to successfully complete the caspase activation cascade downstream of the mitochondria.

Discussion

Although p21 was originally identified as a mediator of p53-induced growth arrest, new functions of p21 emerged recently, including its role as a modulator of apoptosis. However, the mechanisms by which p21 interferes with the death-inducing signaling cascade are still very elusive. Based on recent evidence, p21 could be involved in the modulation of apoptosis at three different levels (13). First, p21 expression was shown to induce genes for secreted proteins with antiapoptotic activities (39). It remains to be tested, however, whether this effect extends beyond the human fibrosarcoma cells in which it was originally described.

Second, by direct binding and inhibition of molecules known to be involved in the apoptotic process, such as caspase-3, JNK, or ASK1 (15–17). To perform those tasks, p21 has to be relocalized to the cytoplasm, which might be achieved either by the caspase-3-mediated cleavage or by its phosphorylation by the kinase Akt (19, 21). In the present report, however, we were not able to document such a cytoplasmic localization of p21 that was strictly confined to the nucleus following exposure of the cells to IR. Consistent with this, also the previously proposed mitochondrial

association of p21 that apparently is required for its binding to procaspase-3 (32) could not be observed in our system. Particularly, a direct binding of p21 to caspase-3 would have been a very attractive model that could have explained the failure of IR to induce apoptosis in HCT116 wild-type cells despite the occurrence of a recently described proapoptotic event (i.e., the translocation of p53 to mitochondria; refs. 4, 5). However, in our hands, neither caspase-3 nor caspase-9 were found to be associated with p21 in IR-treated cells. This suggests that the observed protection by p21 is most likely mediated via other events not involving a cytoplasmic localization or a direct interaction with proapoptotic proteins, such as caspases.

Finally, p21 was postulated to modulate apoptotic processes via inhibition of CDKs. Evidence for such a mechanism was not only obtained by reports showing increased CDK activities in apoptotic cells (23, 24, 40) but also from studies in which the protective function of p21 could be abrogated by deletion of its NH_2-terminal region required for CDK binding (41). However, although inhibition of CDKs by either dominant-negative mutants or pharmacologic inhibitors prevented the generation of several apoptotic characteristics, in most cases, the CDK function was localized downstream of the caspase cascade. In contrast to these studies, we report here for the first time an involvement of CDKs in IR-induced apoptosis acting downstream of the mitochondria, but upstream of caspase-9 activation. These conclusions were drawn from our observation that IR treatment not only resulted in the activation of the mitochondria including cytochrome *c* release in apoptosis-sensitive checkpoint-deficient cells but also in resistant HCT116 wild-type cells in which IR failed to activate caspase-9 and caspase-3. These data were further substantiated by our finding that the selective CDK1/CDK2 inhibitor roscovitine efficiently prevented IR-induced caspase-9 and caspase-3 processing and activation in p21-deficient cells but had no effect on caspase-2 processing or the loss of $\Delta\Psi_m$ induced by this treatment. Inhibition of apoptosis by CDK inhibitors, such as roscovitine or structurally related compounds, was also observed in several other cellular systems; the underlying events that were affected by these treatments, however, were not further investigated (42–44).

In contrast to our study, it was reported previously that only p21-deficient HCT116 cells, but not wild-type cells, displayed extensive $\Delta\Psi_m$ loss, cytochrome *c* release, and caspase activation following treatment with the DNA-damaging agent Adriamycin (45). The exact reason for the discrepancy to our study is presently unknown but is most likely due to stimulus-dependent signaling events (46).

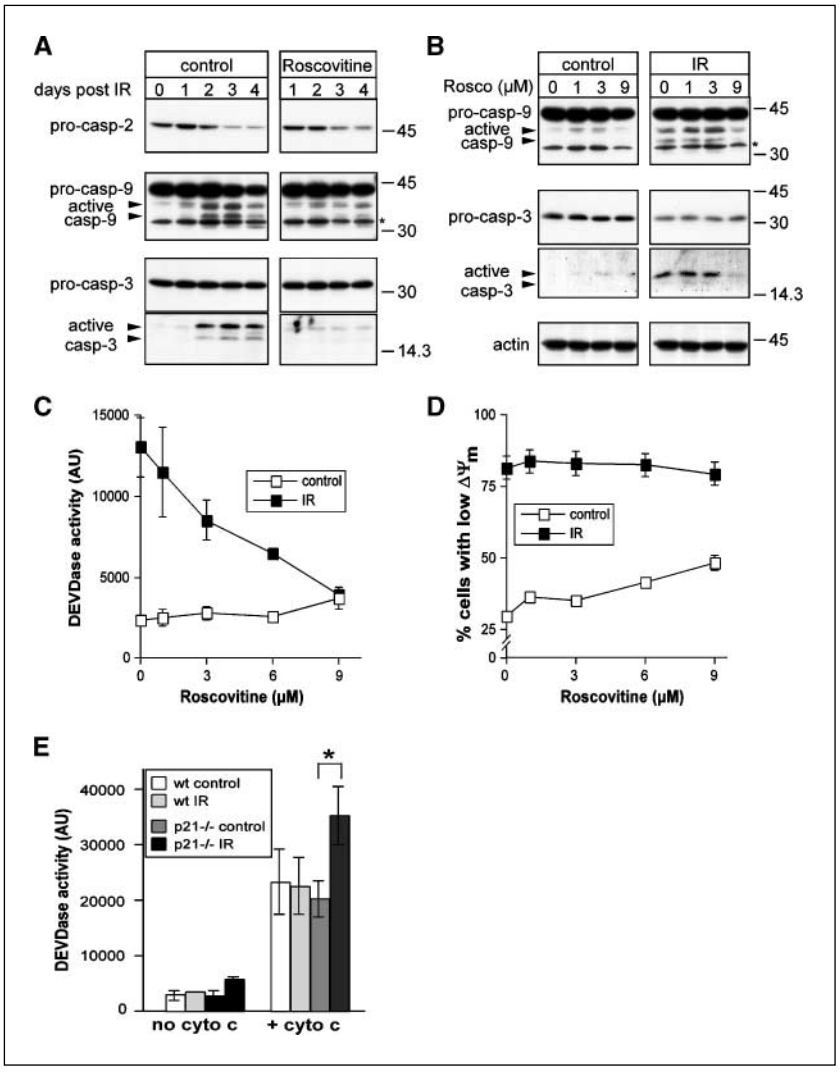

Figure 6. The CDK inhibitor roscovitine prevents processing and activation of caspases downstream of the mitochondria. *A,* Western blot analyses for the status of the indicated caspases in p21-deficient cells that were irradiated and incubated for the indicated times in the absence (control) or presence of 10 μmol/L roscovitine. *B,* Western blot analyses for the status of the indicated caspases in p21-deficient cells that were irradiated and incubated for 3 days in the absence (control) or presence of the indicated roscovitine concentrations. Blots shown in (*A*) and (*B*) are a representative experiment out of two. *Arrows,* active caspases; *, band of unknown origin. *C,* measurement of the caspase-3 (DEVDase) enzymatic activities in extracts of untreated and irradiated p21-deficient cells that were incubated for 2 days after IR in the absence or presence of the indicated concentrations of roscovitine. *D,* measurement of the mitochondrial membrane potential ($\Delta\Psi_m$) of p21-deficient cells incubated for 3 days after irradiation in the absence or presence of the indicated concentrations of roscovitine. *E, in vitro* caspase activation assay. Extracts of untreated and irradiated p21-deficient cells were prepared 1 day after IR and were incubated for 1 hour in the absence or presence of cytochrome *c*/dATP and subsequently analyzed for caspase-3 (DEVDase) enzymatic activities. *Points/columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$ (Student's *t* test).

In further support for this assumption is our finding that caspase-2 is activated by IR also in resistant wild-type cells in the absence of active caspase-9 or caspase-3. Together with the fact that the IR-induced $\Delta\Psi_m$ loss could be partially blocked by z-VDVAD-fmk, a caspase-2-specific inhibitory peptide, our data argue for a pre-mitochondrial role of caspase-2 in IR signaling. In contrast, in drug-induced apoptosis, caspase-2 activation was mainly detected downstream of the mitochondria (47), an observation that might explain the lack of mitochondrial activation in Adriamycin-treated HCT116 wild-type cells. However, whether caspase-2 plays a role upstream or downstream of mitochondria is still controversially discussed (29).

What are the specific CDK-mediated events required for apoptosis induction? Although a recent screen of a yeast proteomic library succeeded in the identification of ~200 CDK substrates (48), only a few human CDK targets have been characterized thus far. This is probably due to the lack of appropriate proteomic libraries and also to the difficulty to identify phosphorylated substrates in a large mixture of proteins. Nevertheless, one of the few CDK targets identified is the proapoptotic Bcl-2 family member BAD that becomes a mitochondrial activator upon phosphorylation by CDK1 (49). However, as we have located the putative CDK-mediated event required for caspase-9 activation downstream of mitochondria, it is unlikely that the phosphorylation of BAD at Ser^{128} plays a role in this system.

Especially with regard to our observation that CDKs act in a post-mitochondrial event, more likely candidates influenced either directly or indirectly by CDK activity might be proteins known to be involved in apoptosome formation, such as APAF-1, the antiapoptotic X-linked inhibitor of apoptosis protein (XIAP), and even caspase-9 itself. In favor of this assumption are our *in vitro* caspase activation experiments in which the formation of the apoptosome is initiated artificially by the addition of cytochrome *c*. Using this assay, we observed consistently a higher inducible caspase-3 activity in extracts of irradiated p21-deficient cells than in extracts of similar treated wild-type cells, suggesting the requirement for a coactivation event that only occurs in the absence of p21. Unfortunately, comparative gel filtration analyses of irradiated cells that would have yielded further insights into the composition of the apoptosome failed. In addition, Western blot analyses for the status of several apoptosome-related proteins, including XIAP and caspase-9, did not reveal any differences with regard to their expression and their migration pattern that would have indicated post-translational modifications (data not shown). Nevertheless, phosphorylation of XIAP and caspase-9 by Akt or other kinases that rendered these cells resistant to apoptosis induction was shown in previous studies (50–52). Furthermore, overexpression of XIAP and/or Akt seems to be a common mechanism to block activation of the caspase cascade downstream of the mitochondria (53), and inhibition of their expression and/or function was recently postulated to overcome chemoresistance in cells expressing wild-type p53 (54). Besides its role as a prosurvival kinase, Akt is also known to control cell cycle progression via stabilization of p21. As pharmacologic CDK inhibitors were shown to modulate Akt activity (26) and possibly also activities of other kinases, these results suggest a complex interaction network between these proteins. Hence, it is tempting to speculate that CDKs interfere with the apoptotic machinery in a similar manner, perhaps by inactivating Akt or other related kinases. This hypothesis is currently under thorough investigation in our laboratory.

In summary, we have shown that the p21-mediated protection against IR-induced apoptosis is most likely caused by inhibition of CDKs that are required for the activation of the caspase cascade downstream of mitochondria. Within this context, it is inevitable to mention that CDK inhibitors such as roscovitine have also recently entered clinical trials not only due to their antiproliferative function but also because of their strong proapoptotic effects that were, however, only observed when higher drug concentrations were used than those applied in the present study. As the outcome of such a therapeutic treatment critically depends on the inhibitor concentrations used, it is absolutely necessary to carefully evaluate the biochemical pathways involved to avoid adverse effects.

Acknowledgments

Received 5/3/2006; revised 8/7/2006; accepted 9/15/2006.

Grant support: Deutsche Forschungsgemeinschaft grant SFB 503 and the Deutsche Krebshilfe.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank B. Vogelstein (The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD) for the HCT116 cell lines, B. Ducommun (Institut d'Exploration Fonctionnelle des Génomes, Université Paul Sabatier, Toulouse, France) for the two DLD1 cell lines expressing either wild-type p21 or a mutant p21 deficient for CDK interaction, and N. Wittig for excellent technical assistance.

References

1. Oren M. Decision making by p53: life, death and cancer. Cell Death Differ 2003;10:431–42.
2. Sax JK, El-Deiry WS. p53 downstream targets and chemosensitivity. Cell Death Differ 2003;10:413–7.
3. Willis SN, Adams JM. Life in the balance: how BH3-only proteins induce apoptosis. Curr Opin Cell Biol 2005;17:617–25.
4. Mihara M, Erster S, Zaika A, et al. p53 has a direct apoptogenic role at the mitochondria. Mol Cell 2003;11:577–90.
5. Essmann F, Pohlmann S, Gillissen B, Daniel PT, Schulze-Osthoff K, Jänicke RU. Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis. J Biol Chem 2005;280:37169–77.
6. Fischer U, Jänicke RU, Schulze-Osthoff K. Many cuts to ruin: a comprehensive update of caspase substrates. Cell Death Differ 2003;10:76–100.
7. Lassus P, Bertrand C, Zugasti O, et al. Anti-apoptotic activity of p53 maps to the COOH-terminal domain and is retained in a highly oncogenic natural mutant. Oncogene 1999;18:4699–709.
8. Essmann F, Engels IH, Totzke G, Schulze-Osthoff K, Jänicke RU. Apoptosis resistance of MCF-7 breast carcinoma cells to ionizing radiation is independent of p53 and cell cycle control but caused by the lack of caspase-3 and a caffeine-inhibitable event. Cancer Res 2004;64:7065–72.
9. Wendt J, Radetzki S, von Haefen C, et al. Induction of p21CIP/WAF-1 and G_2 arrest by ionizing irradiation impedes caspase-3-mediated apoptosis in human carcinoma cells. Oncogene 2006;25:972–80.
10. Bunz F, Hwang PM, Torrance C, et al. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest 1999;104:263–9.
11. Tian H, Wittmack EK, Jorgensen TJ. p21WAF1/CIP1 antisense therapy radiosensitizes human colon cancer by converting growth arrest to apoptosis. Cancer Res 2000;60:679–84.
12. Waldman T, Zhang Y, Dillehay L, et al. Cell-cycle arrest versus cell death in cancer therapy. Nat Med 1997;3:1034–6.
13. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. Mol Cancer Ther 2002;1:639–49.
14. Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. Cancer Res 2005;65:3980–5.
15. Asada M, Yamada T, Ichijo H, et al. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. EMBO J 1999;18:1223–34.
16. Shim J, Lee H, Park J, Kim H, Choi EJ. A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. Nature 1996;381:804–6.
17. Suzuki A, Tsutomi Y, Akahane K, Araki T, Miura M. Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. Oncogene 1998;17:931–9.
18. Li Y, Dowbenko D, Lasky LA. AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of

p21Cip/WAF1 and promotes cell survival. J Biol Chem 2002;277:11352–61.
19. Zhou BP, Liao Y, Xia W, Spohn B, Lee MH, Hung MC. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol 2001;3:245–52.
20. Blagosklonny MV. Are p27 and p21 cytoplasmic oncoproteins? Cell Cycle 2002;1:391–3.
21. Levkau B, Koyama H, Raines EW, et al. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. Mol Cell 1998;1:553–63.
22. Nakamura K, Arai D, Fukuchi K. Identification of the region required for the antiapoptotic function of the cyclin kinase inhibitor, p21. Arch Biochem Biophys 2004; 431:47–54.
23. Zhou BB, Li H, Yuan J, Kirschner MW. Caspase-dependent activation of cyclin-dependent kinases during Fas-induced apoptosis in Jurkat cells. Proc Natl Acad Sci U S A 1998;95:6785–90.
24. Harvey KJ, Lukovic D, Ucker DS. Caspase-dependent Cdk activity is a requisite effector of apoptotic death events. J Cell Biol 2000;148:59–72.
25. Hakem A, Sasaki T, Kozieradzki I, Penninger JM. The cyclin-dependent kinase Cdk2 regulates thymocyte apoptosis. J Exp Med 1999;189:957–68.
26. Yu C, Rahmani M, Dai Y, et al. The lethal effects of pharmacological cyclin-dependent kinase inhibitors in human leukemia cells proceed through a phosphatidylinositol 3-kinase/Akt-dependent process. Cancer Res 2003;63:1822–33.
27. Waterhouse NJ, Trapani JA. A new quantitative assay for cytochrome *c* release in apoptotic cells. Cell Death Differ 2003;10:853–5.
28. Sohn D, Schulze-Osthoff K, Jänicke RU. Caspase-8 can be activated by interchain proteolysis without receptor-triggered dimerization during drug-induced apoptosis. J Biol Chem 2005;280:5267–73.
29. Zhivotovsky B, Orrenius S. Caspase-2 function in response to DNA damage. Biochem Biophys Res Commun 2005;331:859–67.
30. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
31. Lakhani SA, Masud A, Kuida K, et al. Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. Science 2006;311:847–51.
32. Suzuki A, Tsutomi Y, Yamamoto N, Shibutani T, Akahane K. Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death. Mol Cell Biol 1999;19:3842–7.
33. Sohn D, Totzke G, Essmann F, Schulze-Osthoff K, Levkau B, Jänicke RU. The proteasome is required for rapid initiation of death receptor-induced apoptosis. Mol Cell Biol 2006;26:1967–78.
34. Giannakakou P, Robey R, Fojo T, Blagosklonny MV. Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G_1/G_2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. Oncogene 2001;20:3806–13.
35. Miyashita T, Krajewski S, Krajewska M, et al. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression *in vitro* and *in vivo*. Oncogene 1994; 9:1799–805.
36. Blagosklonny MV, Demidenko ZN, Fojo T. Inhibition of transcription results in accumulation of Wt p53 followed by delayed outburst of p53-inducible proteins: p53 as a sensor of transcriptional integrity. Cell Cycle 2002;1:67–74.
37. Cayrol C, Knibiehler M, Ducommun B. p21 binding to PCNA causes G_1 and G_2 cell cycle arrest in p53-deficient cells. Oncogene 1998;16:311–20.
38. Bach S, Knockaert M, Reinhardt J, et al. Roscovitine targets, protein kinases and pyridoxal kinase. J Biol Chem 2005;280:31208–19.
39. Chang BD, Watanabe K, Broude EV, et al. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. Proc Natl Acad Sci U S A 2000;97:4291–6.
40. Shi L, Nishioka WK, Th'ng J, Bradbury EM, Litchfield DW, Greenberg AH. Premature p34cdc2 activation required for apoptosis. Science 1994;263:1143–5.
41. Lu Y, Yamagishi N, Yagi T, Takebe H. Mutated p21(WAF1/CIP1/SDI1) lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. Oncogene 1998;16:705–12.
42. Park DS, Morris EJ, Greene LA, Geller HM. G_1/S cell cycle blockers and inhibitors of cyclin-dependent kinases suppress camptothecin-induced neuronal apoptosis. J Neurosci 1997;17:1256–70.
43. Hsu SL, Yin SC, Liu MC, Reichert U, Ho WL. Involvement of cyclin-dependent kinase activities in CD437-induced apoptosis. Exp Cell Res 1999;252:332–41.
44. Verdaguer E, Jorda EG, Canudas AM, et al. Anti-apoptotic effects of roscovitine in cerebellar granule cells deprived of serum and potassium: a cell cycle-related mechanism. Neurochem Int 2004;44:251–61.
45. Le HV, Minn AJ, Massague J. Cyclin-dependent kinase inhibitors uncouple cell cycle progression from mitochondrial apoptotic functions in DNA-damaged cancer cells. J Biol Chem 2005;280:32018–25.
46. Jänicke RU, Engels IH, Dunkern T, Kaina B, Schulze-Osthoff K, Porter AG. Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. Oncogene 2001;20:5043–53.
47. Ruiz-Vela A, Opferman JT, Cheng EH, Korsmeyer SJ. Proapoptotic BAX and BAK control multiple initiator caspases. EMBO Rep 2005;6:379–85.
48. Ubersax JA, Woodbury EL, Quang PN, et al. Targets of the cyclin-dependent kinase Cdk1. Nature 2003;425: 859–64.
49. Konishi Y, Lehtinen M, Donovan N, Bonni A. Cdc2 phosphorylation of BAD links the cell cycle to the cell death machinery. Mol Cell 2002;9:1005–16.
50. Dan HC, Sun M, Kaneko S, et al. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). J Biol Chem 2004;279:5405–12.
51. Martin MC, Allan LA, Lickrish M, Sampson C, Morrice N, Clarke PR. Protein kinase A regulates caspase-9 activation by Apaf-1 downstream of cytochrome *c*. J Biol Chem 2005;280:15449–55.
52. Brady SC, Allan LA, Clarke PR. Regulation of caspase 9 through phosphorylation by protein kinase C zeta in response to hyperosmotic stress. Mol Cell Biol 2005;25: 10543–55.
53. Hajra KM, Liu JR. Apoptosome dysfunction in human cancer. Apoptosis 2004;9:691–704.
54. Fraser M, Leung BM, Yan X, Dan HC, Cheng JQ, Tsang BK. p53 is a determinant of X-linked inhibitor of apoptosis protein/Akt-mediated chemoresistance in human ovarian cancer cells. Cancer Res 2003;63:7081–8.