Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription

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Summary

Recent efforts to develop pharmacologic agents that restore function to mutant forms of p53 hold significant promise in cancer therapy. Here, we examine the effects of such pharmacologic activation of p53 function using a small molecule, PRIMA-1, and a model system employing a p53 protein fused to a mutant steroid binding domain of the murine estrogen receptor (p53ER^{tam}) that renders it responsive only in the presence of 4-hydroxytamoxifen. In either case, p53 activation triggered apoptosis that was not inhibited by the presence of macromolecular synthesis inhibitors. This p53-induced, transcription-independent apoptosis is Bax dependent, proceeds in the absence of a nucleus, and involves Bax translocation and cytochrome c release. Hence, pharmacologic p53 modulators can activate a transcription-independent apoptotic program.

Introduction

The p53 tumor suppressor integrates cellular stresses, such as growth factor or oxygen deprivation, DNA damage, and oncogenic transformation, to trigger either cell cycle arrest and senescence, DNA repair, or apoptosis (Vogelstein et al., 2000). Thereby, p53 prevents the propagation of cells that have lost their genetic or functional integrity and thus could threaten the organism by cancerous outgrowth. Accordingly, humans and mice with germline defects of the *TP53* gene are prone to early development of tumors (Malkin et al., 1990; Donehower et al., 1992). Further, in murine models, the development of genetically programmed cancers is greatly accelerated on a p53-deficient background (Eischen et al., 1999; Schmitt et al., 1999; Johnson et al., 2001; Van Dyke and Jacks, 2002), and the majority of human cancers show mutations of *TP53* or its regulators (Olivier et al., 2003). In vivo dissection of p53-dependent tumor suppression revealed apoptosis as its main effector mechanism (Schmitt et al., 2002). Hence, tumor cell-specific induction of apoptosis by pharmacologic activation of mutant p53 is an attractive therapeutic and preventive strategy in oncology. A better understanding of the molecular events controlling p53-induced cell death is a prerequisite for such an approach.

Genetic and biochemical evidence suggests that p53 engages apoptotic caspase activation mainly through the intrinsic, "mitochondrial" pathway (Schuler and Green, 2001). This pathway is regulated by various pro- and antiapoptotic BCL-2 family proteins, which govern the integrity of the mitochondrial membranes (Green and Evan, 2002; Adams and Cory, 1998). Mitochondrial outer membrane permeabilization (MOMP) results in the liberation of apoptogenic factors, including cytochrome c, SMAC/DIABLO, HtrA2/Omi, AIF, or EndoG, which facilitate caspase activation or nuclear DNA fragmentation (Green, 2000). Accordingly, p53 was shown to induce cytochrome c release and caspase activation via proapoptotic BCL-2 proteins (Schuler et al., 2000, 2003; Zhang et al., 2000), and fibroblasts from mice deficient in BAX, APAF-1, or caspase-9 were resistant to p53-dependent cell death (McCurrach et al., 1997; Soengas et al., 1999).

It is thought that p53 signals apoptosis through its activity as a sequence-specific transcriptional activator of target genes, such as *BAX*, *NOXA*, *PUMA*, *BID*, *APAF-1*, *CD95*, *DR5*, or *p53AIP1* (Miyashita and Reed, 1995; Wu et al., 1997; Müller et al., 1998; Oda et al., 2000a, 2000b; Yu et al., 2001; Nakano and Vousden, 2001; Moroni et al., 2001; Sax et al., 2002). Accordingly, pharmacologic strategies to activate mutant p53 have focused on the identification of compounds that restore its capacity to bind specific DNA sequences (Wieczorek et al., 1996; Selivanova et al., 1997; Foster et al., 1999; Bykov et al., 2002).

In addition to transactivation of target genes, evidence from several experimental systems and laboratories has suggested a contribution of transcription-independent components to p53's

SIGNIFICANCE

The p53 tumor suppressor is encoded by the most commonly mutated gene in cancer, and its role in preventing oncogenesis through the induction of apoptosis is well established. While most functions of p53 involve its activity as a transcription factor, evidence has also implicated transcription-independent activities in its induction of apoptosis. Pharmacologic approaches to restore p53 function have focused on the reestablishment of transcriptional activity, but here we show that such restoration triggers Bax-dependent apoptosis involving cytochrome c release and caspase activation in the absence of transcription (or, indeed, a nucleus). Therefore, approaches for the restoration of this transcription-independent proapoptotic activity of p53 are viable therapeutic strategies.

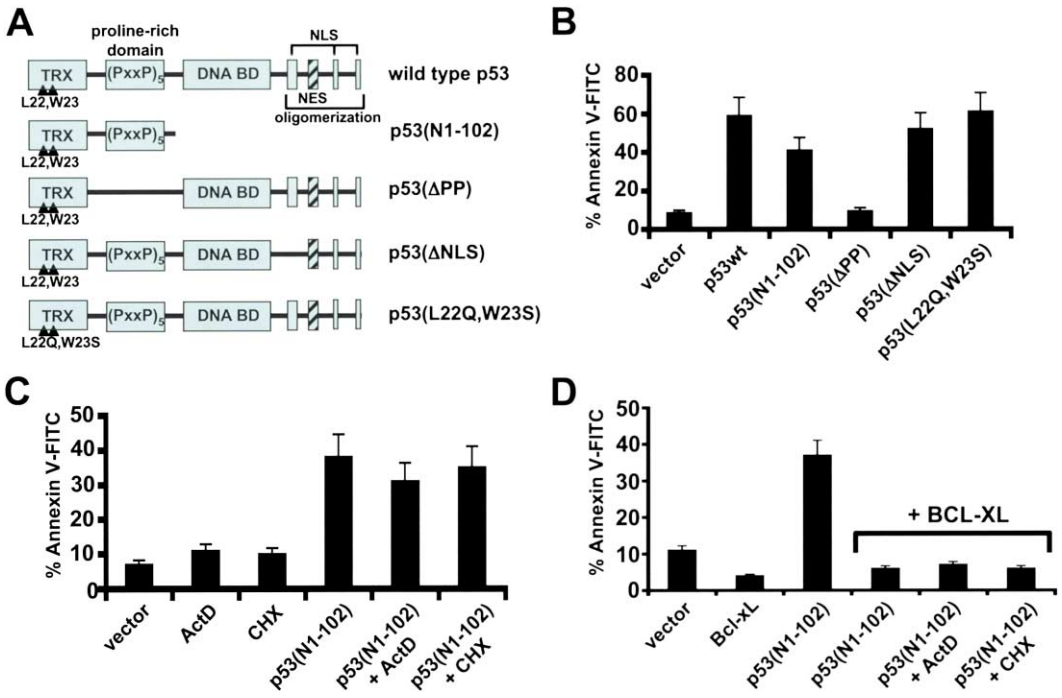

Figure 1. p53-induced apoptosis in the absence of DNA binding, transcription, and translation is blocked by BCL-XL

A: Schematic representation of the p53 domains and mutants expressed in NCI-H1299 cells.
B: NCI-H1299 cells were transiently transfected with indicated p53 vectors and analyzed 48 hr after by Annexin V-FITC staining and FACS.
C: NCI-H1299 were transiently transfected with $p53^{N1-102}$, treated with 10 nM actinomycin D or 10 μg/ml cycloheximide (or vehicle), and analyzed 48 hr after by Annexin V-FITC staining and FACS.
D: NCI-H1299 were transfected and treated as in **C**, but BCL-XL was added where indicated. Data are representative of three independent experiments. Error bars equal ± SD.

proapoptotic activity (Caelles et al., 1994; Wagner et al., 1994; Haupt et al., 1995; Chen et al., 1996). Modulation of the localization of death receptors, a requirement for caspase-8, as well as a direct impact of p53 on mitochondria have been proposed as transcription-independent apoptotic mechanisms (Bennett et al., 1998; Marchenko et al., 2000; Dumont et al., 2003; Mihara et al., 2003; Ding et al., 2000). As the vast majority of naturally occurring p53 mutations target the DNA binding domain and thereby abolish its activity as a transcriptional activator, we reasoned whether activation of p53's transcription-independent proapoptotic activity might be a feasible therapeutic strategy. To this end, we have established experimental systems to define and validate the p53 domain crucial for transcription-independent apoptosis, as well as to dissect this activity at a molecular level. We provide models for the "pharmacologic activation" of p53 at the cellular and subcellular levels by the use of an inducible p53 fusion protein and a small molecular compound capable of activating wild-type or mutant p53, and in both cases, we find that apoptosis can be induced in a manner that is p53 dependent but transcription *in*dependent.

Results

The amino terminus of p53 including its proline-rich regulatory domain is necessary and sufficient to induce transcription-independent apoptosis

Extensive characterization has led to the definition of functional domains of the p53 tumor suppressor protein, which are summarized in Figure 1A (Levine, 1997; Vogelstein et al., 2000). The integrity of the central DNA binding domain was shown to be essential for p53's function as a transcriptional activator, and the majority of p53 mutations found in cancer target this domain (Olivier et al., 2003). Additional domains, such as the amino-terminal proline-rich domain, were shown to be important for transrepression and induction of apoptosis (Walker and Levine, 1996; Sakamuro et al., 1997; Venot et al., 1999). Recently, it was suggested that transcription-independent induction of apoptosis by p53 also required the integrity of the DNA binding domain (Mihara et al., 2003). However, the same group previously reported that the core domain mutant $p53^{R175H}$ was still capable of inducing apoptosis when targeted to the mitochondria (Marchenko et al., 2000). In order to define minimal requirements for p53-induced transcription-independent apoptosis, we expressed various artificial p53 mutants (Figure 1A) in NCI-H1299 lung cancer cells (Figure 1B) and Saos-2 osteosarcoma cells (not shown). These studies confirmed that a transactivation domain mutant $p53^{L22Q,W23S}$ was still capable of inducing apoptosis (Haupt et al., 1995). Similarly, a $p53^{\Delta NLS}$ mutant with a destroyed nuclear localization signal, which is targeted to the cytoplasm (not shown), induced apoptosis in this assay (Figure 1B). In contrast, a $p53^{\Delta PP}$ mutant lacking the amino-terminal proline-rich regulatory domain (residues 62 to 91) (Walker and Levine, 1996; Sakamuro et al., 1997) exhibited no proapoptotic activity (Figure 1B). A $p53^{N1-102}$ truncation mutant, which encompasses the proline-rich domain but not the DNA binding and carboxy-

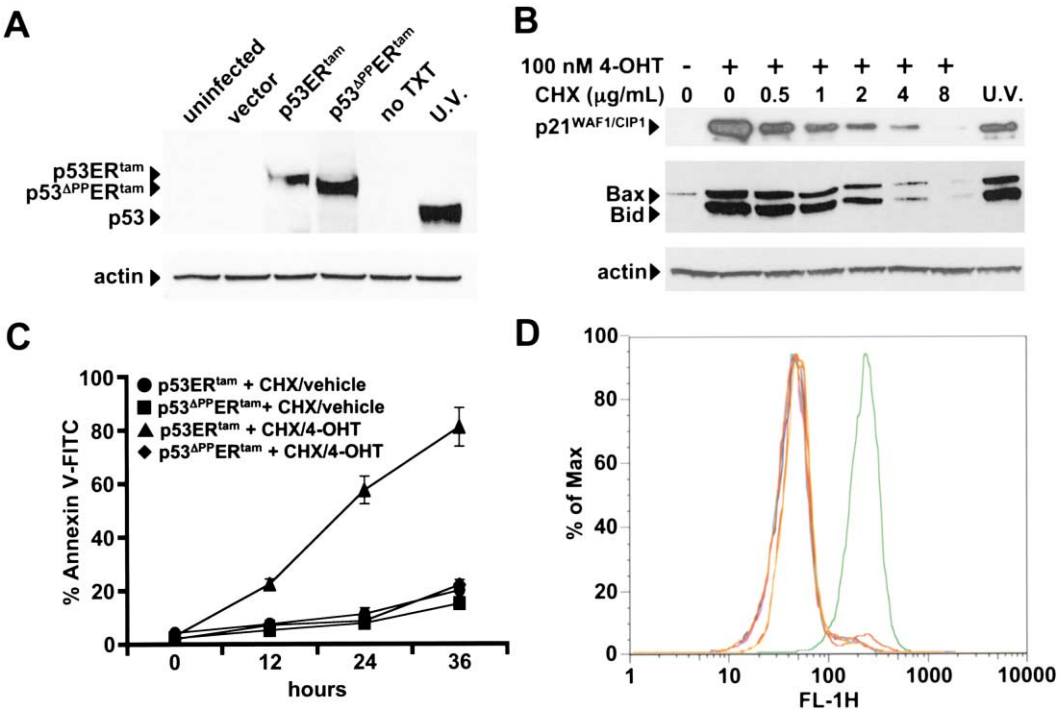

Figure 2. Transcription-independent, p53ER^{tam}-induced apoptosis occurs independently of de novo protein synthesis and CD95/Fas/APO-1 redistribution

A: The stable expression of p53ER^{tam} and p53$^{\Delta PP}$ER^{tam} in NCI-H1299 is equal to UV-induced expression of endogenous p53 in HCT116 cells. Both exogenous and endogenous p53 were detected by Western blot analysis with anti-p53 clone Do7. Actin is shown as a loading control.

B: p53ER^{tam}-induced gene expression in NCI-H1299 is inhibited by cycloheximide. Cells were cultured with indicated concentrations of cycloheximide (or vehicle) for 60 min prior to treatment with 100 nM 4-OHT (or vehicle) for 24 hr before Western blot analysis with anti-p21$^{WAF1/CIP1}$, Bax, and Bid. The blot was not stripped of anti-Bax antibody before probing for Bid. Actin is shown as a loading control.

C: p53ER^{tam}-induced apoptosis occurs without de novo protein synthesis and requires the p53 proline-rich domain (amino acids 62–91) in NCI-H1299 cells. Cells were cultured with 10 μg/ml cycloheximide (or vehicle) for 60 min prior to treatment with 100 nM 4-OHT (or vehicle) for 48 hr. Apoptosis was measured by Annexin V-FITC staining and FACS.

D: CD95/Fas/APO-1 is not redistributed to the cell surface during p53ER^{tam}-induced death. NCI-H1299 were cultured with 10 μg/ml cycloheximide for 60 min prior to treatment with 100 nM 4-OHT. Cells were harvested at indicated time points, stained with anti-CD95/Fas/APO-1-FITC before FACS analysis. Overlapping data points are 0 min, 30 min, 60 min, 120 min, 12 hr of 4-OHT treatment, and a CD95/Fas/APO-1 negative control. The FL1-positive peak is a CD95/Fas/APO-1 overexpressing cell line positive control. Data are representative of several independent experiments. Error bars equal ± SD.

terminal domains, still exhibited a profound and reproducible proapoptotic activity (Figure 1B). In order to exclude residual effects of the p53^{N1-102} mutant on target gene induction, NCI-H1299 cells transfected with this mutant were then additionally treated after 24 hr with inhibitors of transcription and translation, actinomycin D and cycloheximide (CHX) (Figure 1C). While this treatment does not exclude gene expression effects during the expression of the transfected mutant, it should have prevented late events that might be rate limiting for apoptosis. We and others have previously shown that p53-dependent apoptosis mainly proceeds through the intrinsic, mitochondrial pathway, which is regulated by the BCL-2 family of proteins (Soengas et al., 1999; Schuler et al., 2000, 2003). Accordingly, transcription-independent apoptosis induced by p53^{N1-102} was effectively blocked by antiapoptotic BCL-XL, either in the absence or presence of actinomycin D or CHX (Figure 1D). In summary, the amino terminus of p53 confers a proapoptotic activity, which depends on the proline-rich regulatory domain and which does not require transactivation of target genes.

As overexpression studies can be prone to nonspecific effects, we made use of a pharmacologically regulated p53 construct (Vater et al., 1996), which we stably expressed in cancer cell populations at levels comparable to those of endogenous p53 following DNA damage (Figure 2A) or expression of oncogenes (Supplemental Figure S1 at http://www.cancercell.org/cgi/content/full/4/5/371/DC1). Both the wild-type p53 and p53$^{\Delta PP}$ cDNA were fused to the mutant ligand binding domain of the murine estrogen receptor, which renders the resulting fusion protein inactive potentially through complex formation with HSP90. Addition of the specific ligand 4-hydroxytamoxifen (4-OHT) releases this complex, resulting in an active fusion protein (Littlewood et al., 1995). This system was devised to model pharmacologic modulation of p53 activity. 4-OHT treatment of cells stably expressing p53ER^{tam} (Figure 2B) as well as p53$^{\Delta PP}$ER^{tam} (data not shown) induced the p53 effector proteins p21$^{WAF1/CIP1}$, Bax, and Bid (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Miyashita and Reed, 1995); and this effect was suppressed by pretreatment with CHX. In contrast, activation of p53ER^{tam}, but not p53$^{\Delta PP}$ER^{tam}, induced apoptosis in NCI-H1299 cells, and this was not inhibited by pretreatment with CHX (Figure 2C). Comparable results were obtained in several additional cell lines, including Saos-2, Jurkat, Molt4,

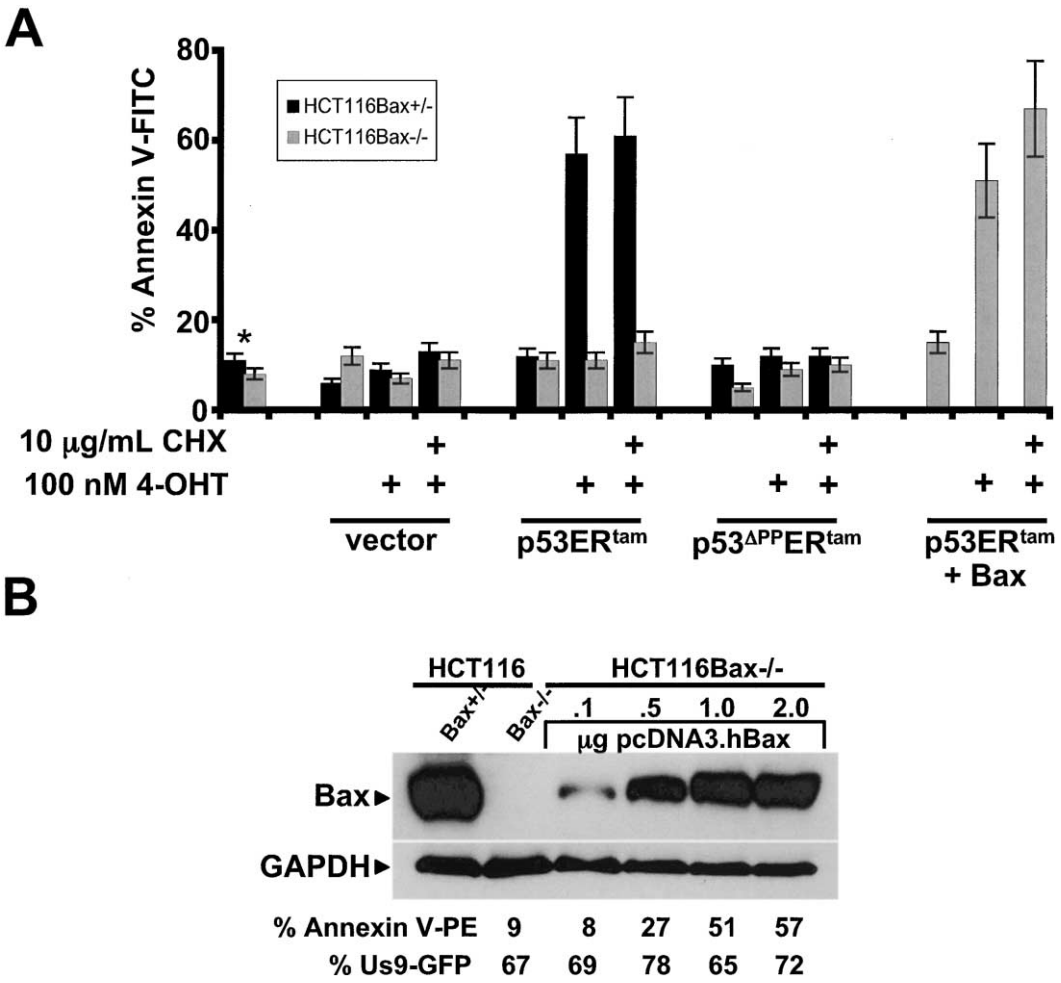

Figure 3. BAX is required for transcription-independent, p53-induced apoptosis

A and **B:** HCT116BAX$^{+/-}$ and HCT116BAX$^{-/-}$ were transiently transfected with p53ER^{tam} or p53$^{\Delta PP}$ER^{tam}, cultured with 5 μg/ml cycloheximide (or vehicle) for 60 min, and treated with 100 nM 4-OHT (or vehicle) for 48 hr before Annexin V-FITC staining and FACS. Where indicated, a sublethal dose of BAX was cotransfected. To determine the sublethal transfection dose of BAX, HCT116BAX$^{-/-}$ were transiently transfected with increasing doses of BAX and Us9-GFP, a marker for transfection efficiency, for 36 hr and analyzed by FACS for % transfected (GFP positive) and % apoptosis (Annexin V-PE). Western blot analysis was performed to ensure appropriate BAX expression in the HCT116 cell lines before and after transfection. GAPDH is shown as a loading control. * designates untransfected cells. Error bars equal ± SD.

HCT116, and oncogene-expressing murine fibroblasts (Figure 3A and data not shown). Using a similar system and vascular smooth muscle cells, Bennett et al. suggested redistribution of the CD95/Fas/APO-1 receptor as the mechanism of p53-induced transcription-independent apoptosis (Bennett et al., 1998). However, we failed to observe a detectable redistribution of CD95/Fas/APO-1 following p53ER^{tam} activation in our experimental system (Figure 2D).

Despite our results that transient expression of p53^{L22Q,W23S} is competent to induce apoptosis (Figure 1B), an in vivo mouse model of the equivalent murine mutant (Trp53^{QS}) indicates that this p53 mutant is exclusively nuclear and does not shuttle to the cytoplasm as wild-type p53 (Jimenez et al., 2000). We confirmed these results (Supplemental Figure S2 on *Cancer Cell* website) and concluded that cytoplasmic effects of the endogenous p53 mutant would therefore not be observed in our system. Furthermore, we speculate that transactivation-deficient (e.g., Trp53^{QS}) and/or DBD mutant (e.g., p53^{I254D}) forms of p53 may lack cytoplasmic proapoptotic activity not only due to the loss of transcriptional activity, but also due to the altered localization (Supplemental Figure S2 online); i.e., enhanced nuclear accumulation that may result from the loss of MDM2 regulation.

Recently, we have shown that during p53-dependent apoptosis, BAX translocates from the cytoplasm to mitochondria, where it induces the release of apoptogenic factors including cytochrome c (Schuler et al., 2003). These same events were observed following 4-OHT activation of p53ER^{tam} in the presence of CHX (Supplemental Figure S3 on *Cancer Cell* website), demonstrating in our experimental system that p53-dependent apoptosis in the absence of translation involves the activation of the mitochondrial pathway of caspase activation. In summary, pharmacologic activation of p53ER^{tam} stably expressed at physiological levels was sufficient to induce apoptosis in a transcrip-

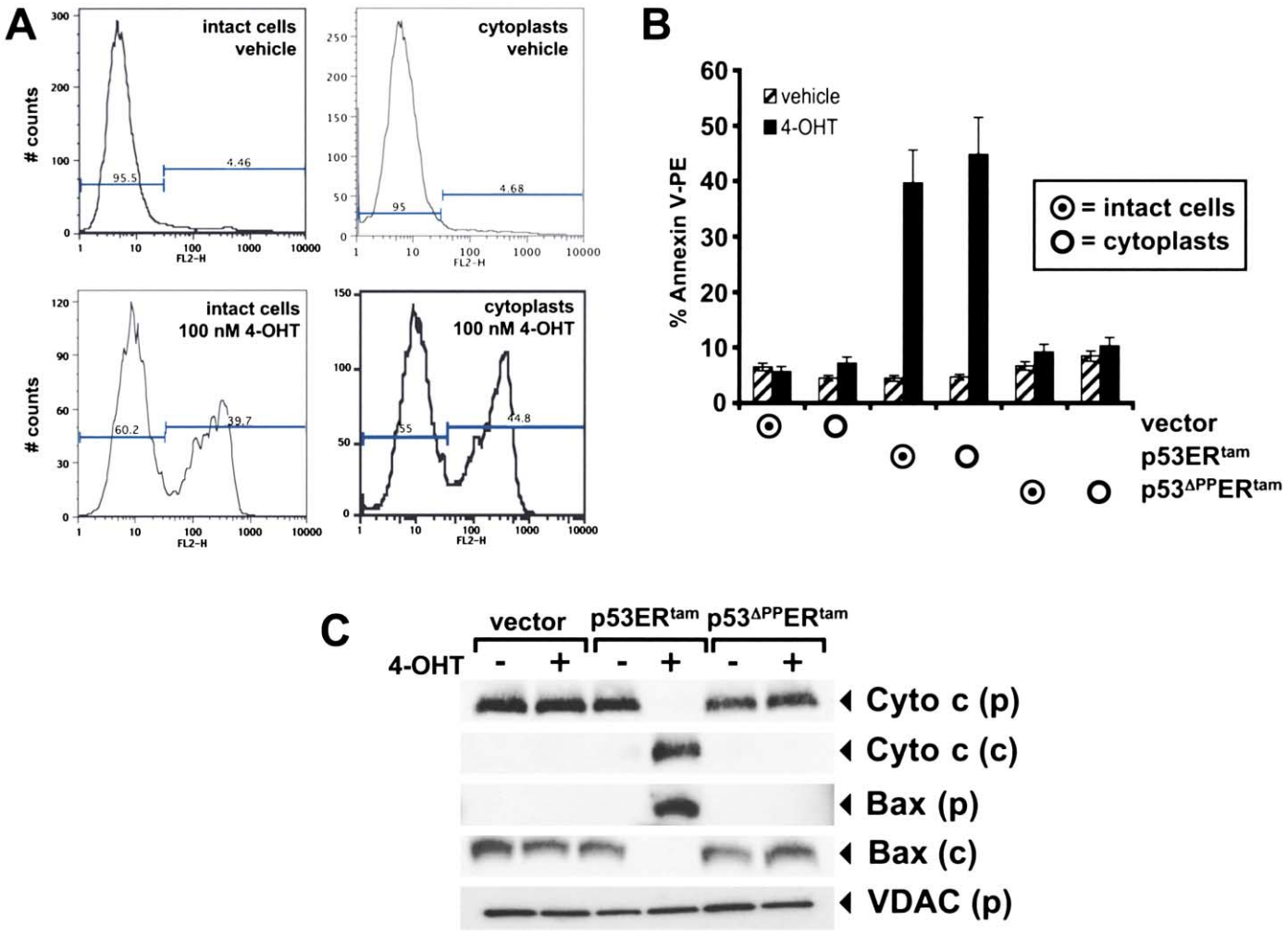

Figure 4. Pharmacological activation of p53 causes BAX translocation, cytochrome c release, and phosphatidylserine externalization in nuclei-free cytoplasts

A: p53ER^{tam}-H1299 cells and cytoplasts were plated 5 hr before a 60 min pretreatment with 10 μg/ml cycloheximide and 100 nM 4-OHT for 36 hr. Intact cells and cytoplasts were then harvested, washed once with PBS, and stained with Annexin V-PE before FACS analysis.

B: Same methods as in **A**, except p53$^{\Delta PP}$ER^{tam} -H1299 cytoplasts were also prepared and analyzed for apoptosis.

C: Cytoplasts were prepared, distinguished, and separated from intact cells by staining with acridine orange and FACS. The pure cytoplast population (acridine orange negative) was cultured for 5 hr, pretreated with 10 μg/ml cycloheximide for 60 min, and treated with 100 nM 4-OHT (or vehicle) for 36 hr. Cytoplasts were then harvested, and the mitochondrial and S-100 fractions were isolated. Western blot analysis comparing the localization of cytochrome c and Bax was performed. VDAC is shown as a loading control for the mitochondrial pellet. (p) and (c) indicate mitochondrial pellet and cytosol, respectively. Data are representative of two independent experiments.

tion-independent manner. Again, this proapoptotic activity of p53 depended on the proline-rich regulatory domain.

Transcription-independent apoptosis of cancer cells by pharmacologically activated p53 requires proapoptotic BAX

The MOMP to release apoptogenic factors, such as cytochrome c, is regulated by the pro- and antiapoptotic BCL-2 family of proteins (Green and Evan, 2002). Previously, we and others have shown that proapoptotic BAX is a crucial mediator of p53-dependent apoptosis in cancer cell lines and murine fibroblasts (McCurrach et al., 1997; Schuler et al., 2000, 2003; Zhang et al., 2000). To study if BAX is required for transcription-independent induction of apoptosis in the present experimental system, we expressed the p53ER^{tam} and p53$^{\Delta PP}$ER^{tam} fusion proteins in HCT116 colorectal cancer cells with heterozygous or homozygous deletions of the BAX gene (Zhang et al., 2000). While 4-OHT activation of p53ER^{tam} but not p53$^{\Delta PP}$ER^{tam} readily induced apoptosis in BAX-proficient HCT116, loss of BAX completely protected HCT116 cells against p53-induced apoptosis in the absence or in the presence of the translation inhibitor CHX (Figure 3A). Sensitivity toward p53-mediated apoptosis was restored by reexpression of sublethal levels of BAX in BAX-deficient HCT116 cells (Figures 3A and 3B). Hence, transcription-independent, p53-mediated apoptosis involves the activation of proapoptotic BCL-2 family proteins, such as BAX, to induce MOMP (Saito et al., 2000; Wei et al., 2000; Kuwana et al., 2002).

Pharmacologically activated p53 induces apoptosis through the mitochondrial pathway in nuclei-free cytoplasts

As we could not formally exclude the induction of an unidentified proapoptotic p53 target protein in the presence of CHX, we set out to study the apoptotic activity of p53ER^{tam} in the absence of nuclei, thus eliminating any possibility of p53-mediated transactivation or transrepression of genes. Previously, it has been shown that apoptosis can proceed in enucleated cells (cytoplasts). Cytoplasts undergoing apoptosis display the same features, such as caspase activation, phosphatidylserine exposure, and cleavage of caspase substrates, that are observed during apoptosis of intact cells (Jacobsen et al., 1994; Martin et al., 1996). To this end, we prepared cytoplasts from cells stably expressing physiological levels of the p53ER^{tam} or the p53$^{\Delta PP}$ER^{tam} fusion proteins (Supplemental Figure S4 on *Cancer Cell* website). When 4-OHT was added to NCI-H1299 cytoplasts expressing p53ER^{tam}, apoptotic membrane changes occurred at the same rate and extent as in intact cells (Figure 4A). Moreover, 4-OHT sensitized p53ER^{tam}-expressing cytoplasts to staurosporine-induced apoptosis (Supplemental Figure S5A online). The proapoptotic activity of p53 in cytoplasts also required the proline-rich regulatory domain (Figure 4B and Supplemental Figure S5A). As in intact cells (Supplemental Figure S3B), p53-dependent apoptosis of cytoplasts proceeded via the mitochondrial pathway, as evidenced by BAX translocation, release of mitochondrial cytochrome c (Figure 4C), and loss of the mitochondrial transmembrane potential $\Delta\psi_m$ (Supplemental Figure S5B) following 4-OHT activation of p53ER^{tam}. The induction of apoptosis in nuclei-free cytoplasts unambiguously demonstrates a cytoplasmic proapoptotic activity of the p53 tumor suppressor protein, which depends on its amino-terminal proline-rich regulatory domain.

Pharmacologically activated p53 induces rapid cytochrome c release from isolated mitochondria in a cell-free reconstituted system

Previously, it has been shown that p53 could directly impact on mitochondria to induce transcription-independent apoptosis. A mtHsp70/Grp75-dependent import of p53 into mitochondria (Marchenko et al., 2000; Dumont et al., 2003), as well as complex formation of p53 with antiapoptotic BCL-2 and BCL-XL (Mihara et al., 2003), were suggested as transcription-independent proapoptotic mechanisms. To further study the potential impact of pharmacologically activated p53 on mitochondria, we developed a cell-free system using cytosolic extracts prepared from cell populations stably expressing physiological levels of the p53ER^{tam} or p53$^{\Delta PP}$ER^{tam} fusion proteins. These cytosolic extracts were incubated with freshly isolated murine liver mitochondria in the presence or absence of 4-OHT. Only p53ER^{tam}, and not p53$^{\Delta PP}$ER^{tam}, could be activated by 4-OHT to induce cytochrome c release from isolated mitochondria (Figure 5). Cytochrome c release was inhibited by the addition of recombinant BCL-XL, which again supports the regulation of this process by the BCL-2 family of proteins (Figure 5). Moreover, the p53-induced release of cytochrome c proceeded independently of caspase activity, as the addition of zVAD-fmk had no effect (Supplemental Figure S6 on *Cancer Cell* website), suggesting that p53 acts upstream of mitochondria to regulate BCL-2 proteins and mitochondrial function. This is consistent with our previous observations in cells and cytosols prepared from cells overexpressing p53 (Schuler et al., 2000).

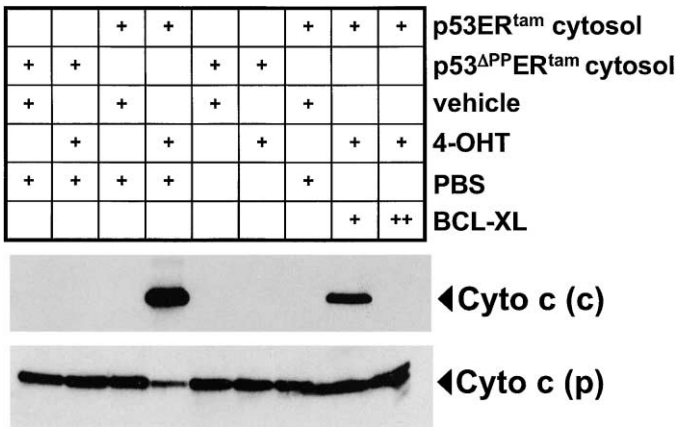

Figure 5. Pharmacological activation of p53 causes cytochrome c release in vitro

S-100 cytosol from p53ER^{tam}-H1299 or p53$^{\Delta PP}$ER^{tam} -H1299 was combined with freshly isolated murine liver mitochondria in the presence of 100 nM 4-OHT (or vehicle). Recombinant BCL-XLΔC (or PBS) was added where indicated. Western blot analysis comparing cytochrome c localization was performed. (p) and (c) indicate mitochondrial pellet and cytosol, respectively. Data are representative of two independent experiments.

The small drug PRIMA-1 activates mutant p53 to induce transcription-independent apoptosis

Our results in cytoplasts and in the cell-free system unambiguously demonstrate that the p53 tumor suppressor protein can elicit a proapoptotic activity that does not depend on an intact DNA binding domain, but on the amino-terminal proline-rich domain. Recently, several small molecular compounds were identified that allow pharmacologic inhibition (Komarov et al., 1999) of wild-type p53 or activation of mutant p53 (Selivanova et al., 1997; Foster et al., 1999; Bykov et al., 2002). The latter compounds were thought to achieve tumor suppression by modulating the capacity of mutant p53 to bind DNA and thereby restoring its activity as a transactivator (Wieczorek et al., 1996; Selivanova et al., 1997; Foster et al., 1999; Bykov et al., 2002). In light of our present observations, it appears possible that compounds modulating the conformation and activation status of p53 DNA binding domain (DBD) mutants could also activate p53 transcription-independent proapoptotic activity.

Therefore, we studied the influence of the small drug PRIMA-1 (Bykov et al., 2002) in our experimental system of p53-induced, transcription-independent apoptosis in a panel of human non-small cell lung carcinoma cell lines with defined p53 status. The cell lines NCI-H460, NCI-H23, and NCI-H1299 (p53$^{wild\text{-}type}$, p53^{M246I}, and p53$^{-/-}$, respectively) were cultured in 20 μg/ml CHX for 30 min prior to treatment with 40 μM PRIMA-1. PRIMA-1 induced rapid apoptosis in cells expressing either p53$^{wild\text{-}type}$ or p53^{M246I} regardless of protein synthesis (Figure 6A), while no effect of PRIMA-1 was observed in the p53$^{-/-}$ cells. Furthermore, the induction of apoptosis by PRIMA-1 proceeds through a caspase-dependent pathway, as inhibition of caspase activity by zVAD-fmk completely abrogated death.

To rule out occult transcription/translation during PRIMA-1-induced death, cytoplasts were prepared from NCI-H1299 cells

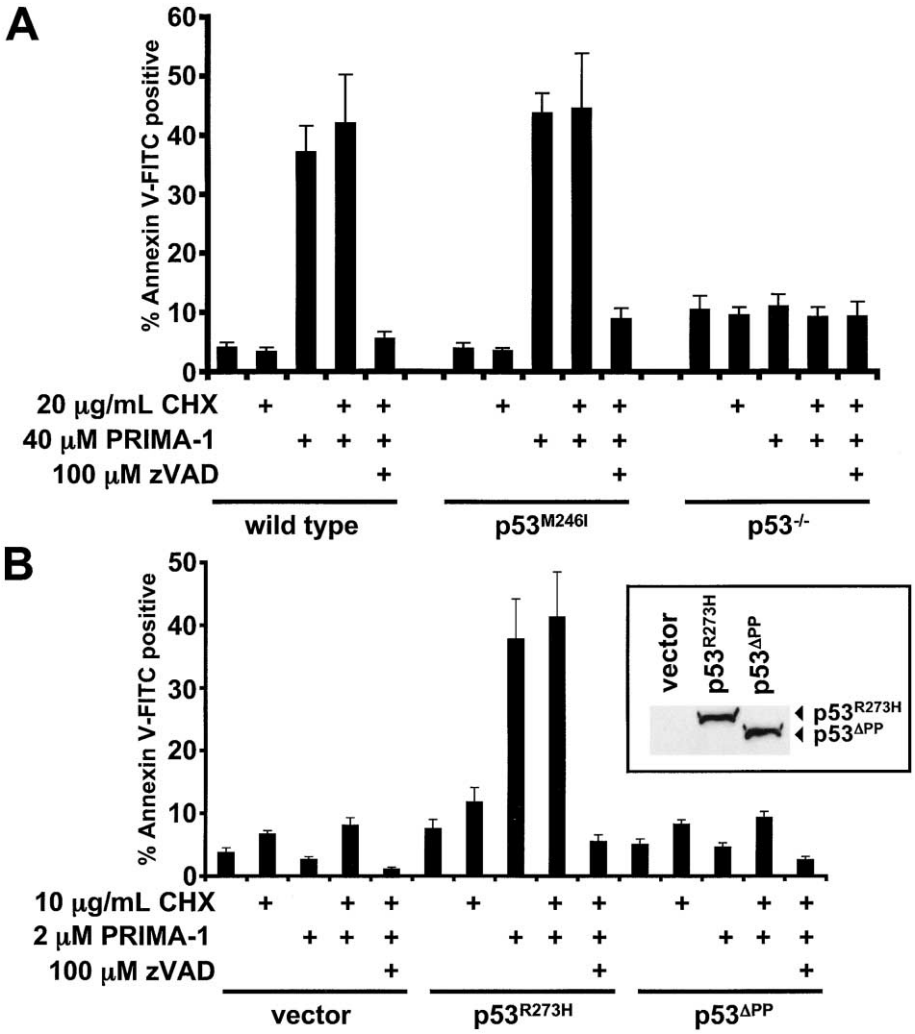

Figure 6. Activation of p53 by PRIMA-1 induces apoptosis in the absence of de novo protein expression or a nucleus

A: NCI-H460, NCI-H23, and NCI-H1299 ($p53^{wt}$, $p53^{M246I}$, $p53^{-/-}$, respectively) human non-small cell lung carcinoma cells lines were pretreated with 20 μg/ml cycloheximide for 30 min prior to treatment with 40 μM PRIMA-1 (or DMSO). Where indicated, 100 μM zVAD-fmk (or DMSO) was added as indicated. Cells were harvested 24 hr after treatment and stained with Annexin V-FITC before FACS analysis. Error bars equal ± SD.

B: Cytoplasts were prepared from empty vector, $p53^{R273H}$ or $p53^{\Delta PP}$ transfected NCI-H1299 cells. After preparation, the cytoplasts were cultured for 8 hr, pretreated with 10 μg/ml cycloheximide and/or 100 μM zVAD-fmk for 60 min, and 2 μM PRIMA-1 for 36 hr. DMSO was added as vehicle for PRIMA-1 and zVAD-fmk. Cytoplasts were harvested and stained with Annexin V-FITC before FACS analysis. Data are representative of two independent experiments. Error bars equal ± SD. Equal expression of $p53^{R273H}$ and $p53^{\Delta PP}$ in cytoplasts was confirmed by immunoblotting (inset).

equally expressing the DBD mutant $p53^{R273H}$ or the $p53^{\Delta PP}$ mutant (Figure 6B inset) and were treated with PRIMA-1 in the absence or presence of CHX. Interestingly, PRIMA-1 clearly induced apoptosis in cytoplasts expressing $p53^{R273H}$, while $p53^{\Delta PP}$ and control cytoplasts proved resistant (Figure 6B). PRIMA-1-induced apoptosis of $p53^{R273H}$ cytoplasts was blocked by pretreatment with the caspase inhibitor zVAD-fmk, but not by CHX (Figure 6B). This ensured that induction of apoptosis by the combination of PRIMA-1 and $p53^{R273H}$ did not involve the translation of residual RNA, which might still be present in cytoplasts, and that this process requires caspase activity. The lack of PRIMA-1-induced apoptosis in $p53^{\Delta PP}$-expressing cytoplasts supports the specificity of this activity in our system.

Discussion

Deregulation of the p53 tumor suppressor pathway is the most prevalent genetic aberration observed in cancer (Sherr and McCormick, 2002). Accordingly, functional restoration of this pathway is an attractive "universal" therapeutic and preventive strategy in oncology. So far, efforts have focused on somatic gene transfer approaches (Roth et al., 1996; Schuler et al., 1998, 2002; Kuball et al., 2002) and the identification of compounds that restore the DNA binding activity of mutant p53 (Selivanova et al., 1997; Foster et al., 1999; Bykov et al., 2002). These approaches have in common that they rely on the preserved capacity of the target cell to respond with transcriptional activation of tumor-suppressive target genes. Most likely all cancer cells cannot fulfil this prerequisite. In order to bypass this potential limitation but still make use of the unique strategic role of p53 in tumor suppression, we set out to study possible transcription-independent, tumor-suppressive activities of the p53 protein. The induction of apoptosis seems to be the main tumor suppressive activity of p53 in vivo (Schmitt et al., 2002). In general, the execution of apoptosis does not require transcription of new genes and, in fact, can be triggered to proceed in nuclei-free cytoplasts (Jacobsen et al., 1994; Schulze-Osthoff et al., 1994; Martin et al., 1996). These observations prompted us to explore the therapeutic modulation of p53's capacity to induce apoptosis in the absence of transcription.

The engagement of the apoptotic machinery by p53 has been studied using a variety of approaches, which have provided conflicting results regarding the requirement of the intact p53 DNA binding domain or transactivation domain for the pro-apoptotic activity of the molecule (Haupt et al., 1995; Chen et al., 1996; Attardi et al., 1996; Zhu et al., 2000; Chao et al., 2000).

Studies using a temperature-sensitive p53 mutant also revealed contradictory results in different cell lines (Caelles et al., 1994; Sabbatini et al., 1995). All studies failed to provide a mechanism for the potential transcription-independent proapoptotic activity of p53. Recently, it has been established that p53-dependent apoptosis mainly proceeds via the intrinsic, mitochondrial pathway of caspase activation (Schuler and Green, 2001). Intriguing studies by Moll et al. suggest that p53 might directly impact on the mitochondria. First, it was reported that p53 is imported into the mitochondria through a mtHsp70/Grp75-dependent mechanism (Marchenko et al., 2000). Moreover, the mitochondrial localization of p53 was found to be regulated by the amino-terminal polymorphism R72P, which also impacts on the interaction between p53 and its negative regulator MDM2 (Dumont et al., 2003). In the latter study, inhibitors of nuclear export of p53 were shown to prevent apoptosis, thus arguing for an important contribution of extranuclear p53 to the induction of cell death (Dumont et al., 2003). Exactly how mitochondrial p53 would trigger apoptosis remains unclear. It has been proposed that p53 can engage a novel caspase-8-dependent pathway to induce apoptosis in the absence of transcription (Ding et al., 2000). More recently, a direct physical interaction of p53 with antiapoptotic BCL-2 and BCL-XL through its DNA binding domain was demonstrated by coimmunoprecipitation and pull-down experiments (Mihara et al., 2003). In this model, mitochondrial p53 would bind antiapoptotic BCL-2 proteins, thereby liberating proapoptotic proteins such as BAX or BAK to induce MOMP and apoptosis, although this has not been demonstrated. Residues 239 to 248 of the p53 DNA binding domain plus flanking regions were apparently required for this interaction. This model conveniently explains why hot spot mutations of the p53 DNA binding domain not only abrogate transactivation but also transcription-independent induction of apoptosis by p53 (Mihara et al., 2003). However, expression of DNA binding domain mutants, such as $p53^{R175H}$, retargeted to the mitochondria were still able to induce apoptosis (Marchenko et al., 2000). In addition, p53 DNA binding domain mutants have been described that locate to the cytoplasm but still induce apoptosis upon transient expression (Crook et al., 1998).

Our present study reveals that the amino-terminal proline-rich regulatory domain (amino acids 62 to 91) is required for the transcription-independent proapoptotic activity of p53, whereas the DNA binding domain may be dispensable. The necessity of the proline-rich domain for tumor-suppressive and death-promoting functions of p53 has previously been highlighted, although a nuclear function for this domain was suggested (Walker and Levine, 1996; Sakamuro et al., 1997; Venot et al., 1999). Although mutations in this region are not common (Sun et al., 1996), several missense mutations have been identified in a variety of human tumors (Database of germline p53 mutations: http://www.lf2.cuni.cz/projects/germline_mut_p53.htm). Using a p53 fusion protein that can be pharmacologically activated, we now provide evidence in nuclei-free cytoplasts as well as in a novel cell-free system that p53 in fact can induce apoptosis through its proline-rich domain in the absence of transcription. These systems provide a "proof-of-concept" for a therapeutic strategy employing pharmacologic modulation of p53's transcription-independent apoptotic activity. Interestingly, a small molecular compound that was found to restore the DNA binding conformation of mutant p53 (Bykov et al., 2002) also triggered p53-dependent, transcription-independent apoptosis. This supports further testing of the applicability of transcription-independent p53 activation in vivo.

The transcription-independent p53-mediated apoptosis seems to be executed by a pathway similar to the one activated under conditions allowing transcription (Schuler and Green, 2001). The release of cytochrome c from isolated mitochondria proceeds through a caspase-independent mechanism (Supplemental Figure S6 on *Cancer Cell* website), whereas the execution phase of apoptosis (i.e., nuclear fragmentation and phosphatidylserine externalization, Figure 6) requires caspase activity, implicating mitochondria as the central mediators of p53-induced apoptosis. The requirement of BAX and the inhibition by BCL-XL in our system points to an involvement of the BCL-2 family of proteins. The activation of BAX-type proteins through p53 may proceed by displacing them from binding antiapoptotic BCL-2-type proteins, as suggested (Mihara et al., 2003). Alternatively, p53 could directly activate BAX-type proteins through its proline-rich domain in a way analogous to BH3-only members of the BCL-2 family (Huang and Strasser, 2001) or peptides derived from the BH3 domain of BID (Letai et al., 2002). The development of a cell-free system of p53-dependent apoptosis now enables the systematic study of factors contributing to the process of BAX activation by p53.

Despite the conclusive evidence presented here, it remains open why expression of most p53 DNA binding domain mutants fails to induce apoptosis despite the presence of an intact proline-rich domain. The answer to this most likely lies in the complex regulation of the p53 protein in vivo. It was shown that under apoptotic conditions, mitochondrially localized p53 is ubiquitinated (Dumont et al., 2003). Polyubiquitination of p53 in general is mediated by MDM2, a transcriptional target of p53 acting in a regulatory negative feedback loop (Oren, 1999). Hence, nuclear export and thus mitochondrial translocation of endogenous p53 might only be possible if the transcription-dependent p53-MDM2 regulation is maintained (Boyd et al., 2000; Geyer et al., 2000). This would explain why DNA binding domain mutants abolish both transcription-dependent and transcription-independent proapoptotic activities of p53. In addition to ubiquitination, nuclear p53 undergoes a plethora of posttranslational modifications during the cellular stress response (Lohrum and Vousden, 1999), which impact on the conformation and activity of the molecule. Does this put a question mark on the therapeutic strategy to pharmacologically activate mutant p53 to induce transcription-independent apoptosis? The answer most likely is no, as it was shown that mutant p53 not only accumulates in the nucleus, but is also found at the mitochondria (Mihara et al., 2003). Pharmacologic modulation of the active conformation of p53 seems achievable in vitro and in vivo (Selivanova et al., 1997; Foster et al., 1999; Bykov et al., 2002). Based on the present study, more detailed functional analyses of p53's transcription-independent proapoptotic activity can be performed. This will enable novel screening strategies for the identification of new p53 modifiers for prevention and treatment of cancer.

Experimental procedures

Plasmids and vectors

The plasmids pBabePuro.$p53^{\Delta PP}ER^{tam}$ and pcDNA3.1.$p53^{N1\text{-}102}$ were obtained by cloning the PCR-generated cDNA into the respective vectors. All inserts were confirmed by sequencing. The plasmid pBabePuro.$p53ER^{tam}$ was pro-

vided by T. Littlewood; various p53 mutants were gifts from A. Levine and G. Wahl.

Cell culture

NCI-H1299, NCI-H460, NCI-H23 (human non-small cell lung carcinoma), Saos-2 (human osteosarcoma), Molt4 (human acute lymphoblastic leukemia), and mouse embryonic fibroblasts were cultured in Dulbecco's Modified Essential Medium supplemented with 10% dextran/charcoal-treated fetal bovine serum (dcFBS), L-glutamine, penicillin, and streptomycin (100 μM 2-mercaptoethanol was added for $Trp53^{QS}$ MEF). Jurkat (human acute T cell leukemia), L1210 (mouse lymphoma), and HCT116 (human colon carcinoma) were cultured as above except with RPMI and McCoy's 5a base media, respectively. Stable cell lines were generated by retroviral infection with pBabePuro.p53ER^{tam}, pBabePuro.p53$^{\Delta PP}ER^{tam}$, or pBabePuro-expressing virions produced by a standard calcium phosphate DNA coprecipitation transfection method with the Phoenix packaging line (a gift from G. Nolan). Puromycin-resistant clones were isolated and pools of >10 clones were used for each analysis. All transient transfections were performed using Lipofectamine 2000 as indicated by the manufacturer.

Flow cytometry, microscopy, and apoptosis assays

Floating and adherent cells were harvested, washed, and stained with annexin V-FITC (BD Clontech) plus propidium iodide (PI) or by annexin V-PE, and the percentage of apoptotic cells was measured by flow cytometry on a Becton Dickinson FACSCalibur. Alternatively, cells were lysed and stained using a hypotonic buffer (0.1% Triton-100, 0.1% sodium citrate, 50 μg/mL PI) for 30 min on ice to determine the fraction of nuclei with sub-G1 DNA content by flow cytometry and cell cycle analysis. For Fas redistribution, NCI-H1299 and L1210 cells were incubated with anti-Fas-FITC at 37°C for 20 min before FACS analysis. Cell death of cytoplasts was measured by flow cytometry following staining with annexin V-FITC or annexin V-PE (BD Pharmingen). For microscopy, cells and cytoplasts growing on coverslips were stained with Hoechst 33342 and MitoTracker Red (Molecular Probes) and were visualized by fluorescence microscopy using an inverted microscope and a digital imaging system.

Isolation of cytoplasts

Cells were trypsinized, incubated with 21 μM cytochalasin B for 1 hr at 37°C, layered over a discontinuous Ficoll gradient (12.5%/15%/16%/17%/25% in 10% dcFBS/HBSS), and ultracentrifuged for 1 hr at 80,000 × g at 37°C. Cytoplasts (12.5%/15% interface) and nucleoplasts (16%/17% interface) were washed with 10% dcFBS/DMEM and plated before Hoechst 33342 staining. Propidium iodide staining after mild digitonin permeabilization (20 μg/ml in PBS, 10 min on ice) was used to distinguish cytoplasts from intact cells. Cytoplast preparations were used only when enucleation was >50%. For FACS sorting of cytoplasts, enucleated/intact cells populations were stained with acridine orange (800 μg/ml in complete media, 37°C for 30 min) before sorting using FACSDiva.

Immunoblotting

Cytosolic extracts were prepared as previously described (Bossy-Wetzel and Green, 1999; Schuler et al., 2000). In brief, cells were harvested and were incubated in a buffer containing 220 mM mannitol and 60 mM sucrose on ice for 30 min. Then, cells were broken in a glass dounce homogenizer. The homogenates were centrifuged at 18,000 × g for 15 min, and the mitochondria-free supernatants were analyzed. Extracts of the pellets as well as whole-cell extracts were obtained by dissolving in lysis buffer, followed by repetitive vortexing and freeze-thawing. After centrifugation at 18,000 × g, the resulting supernatants were analyzed. Cellular fractionation (Supplemental Figure S2 on *Cancer Cell* website) was performed as described (Nikolaev et al., 2003). For SDS-PAGE, 25 or 50 μg of total protein per lane were loaded on 6% or 15% gels. Primary antibodies were anti-p53 Do7, PAb122 and PAb240, anti-p21/WAF1/CIP1 (clone SX118), anti-Bax (polyclonal rabbit antiserum), anti-cytochrome c (clone 7H8.2C12, all BD Pharmingen), anti-PCNA (PC10, Santa Cruz), anti-VDAC (31HL, Calbiochem), anti-Bid (rabbit polyclonal anti-serum), and anti-actin (clone C4, ICN Pharmaceuticals).

In vitro cytochrome c release assay

NCI-H1299 or Saos-2 cells expressing ER^{tam} fusion proteins were harvested by trypsinization, washed four times with ice-cold PBS, and all residual PBS was removed. Pellets were resuspended in 3 volumes of HB (10 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.67 mM dithiothreitol, 0.67 mM phenylmethylsulfonyl fluoride, and 50 ug/ml each leupeptin, aprotinin, and antipain) and incubated on ice for 20 min. Suspensions were lysed through a 25 gauge needle, centrifuged twice at 12,500 × g for 20 min at 4°C, and recentrifuged at 100,000 × g for 60 min at 4°C. Extracts were then clarified through a 0.2 μm filter and brought to 5% glycerol. Freshly isolated murine liver mitochondria were isolated with mitochondria isolation buffer as described (Finucane et al., 1999; Schuler et al., 2000). For 6xHis-BCL-XL purification, BL-21 cells were grown in 2YT medium to $OD_{400} = 0.6$, induced with 0.4 mM IPTG for 3 hr, and lysates were allowed to bind Ni^{2+} agarose for 2 hr at 4°C. The agarose beads were then washed thrice with PBS, washed twice with PBS + 10 mM imidazol, and eluted with 500 mM imidazol overnight at 4°C before dialysis in PBS. 200 μg of extract was combined with 100 μg mitochondria, 100 nM 4-OHT (or ethanol), 1 μg BCL-XL (or PBS) for 60 min (or as indicated) at 37°C. When indicated, the broad-spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD-fmk, Enzyme System Products) dissolved in DMSO was added at 10 μM at the start of the incubation. Samples were centrifuged at 14,000 × g for 20 min and cytosol and mitochondrial (washed twice with MIB) fractions were combined with SDS loading buffer before SDS-PAGE and immunoblotting.

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