Oncogene-dependent Regulation of Caspase Activation by p53 Protein in a Cell-free System*

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The mechanism by which p53 modulates apoptosis in cancer therapy is incompletely understood. Here, cell-free extracts from irradiated tumor cells are described in which endogenous p53 protein is shown to participate in caspase activation. This apoptotic activity is also oncogene-dependent, but independent of transcription in general or the presence of Bax or cytochrome c. A general use for this system is as a cell-free screen for apoptosis modulators. In this way, profound effects of protein kinase A were identified and corroborated in vivo by the protection conferred by cAMP against diverse triggers of p53-dependent apoptosis. This system provides direct biochemical evidence that p53 protein can transduce apoptotic signals through protein-protein interactions and reveals a modulator kinase pathway capable of regulating p53-dependent caspase activation.

p53 plays a major role in modulating the apoptotic response in tumor cells following signals such as DNA damage, growth factor deprivation, and hypoxia (1–3). Its regulation of apoptosis in the context of oncogene overexpression suggests that apoptosis constitutes an important component of p53's tumor suppressor activity. In transgenic mice inactivation of p53, either by germ-line mutation or by expression of the p53-binding domain of SV40 large T antigen leads to attenuation of apoptosis and rapid tumor progression (4). p53-mediated apoptosis has also been implicated as an important mechanism by which many antitumor treatments kill cancer cells (5, 6). Thus, studies of p53-mediated apoptosis may have important implications for cancer treatment.

The mechanism by which p53 modulates apoptosis is incompletely understood. Cell-based studies have provided evidence that p53 can induce apoptosis through either transcription-dependent or transcription-independent mechanisms (1–3). In the transcription-dependent pathway(s), the pro-apoptotic proteins Bax, the insulin-like growth factor-binding protein-3, the death receptor KILLER/DR5, the zinc finger protein PAG608, and proteins involved in generation or response to oxidative stress are potentially important transcriptional targets of p53 (7–12). Little is currently known about the mechanistic basis for p53-mediated apoptosis that is independent of transcription, which may be part of the transcription-independent activities of p53 involved in tumor suppression (13–15).

A cell-free p53-regulated apoptosis system would be useful for biochemically dissecting this medically important pathway. The possibility that a cell-free system may be able to recapitulate p53-dependent apoptosis is suggested by evidence that p53 can induce apoptosis independent of its transcription function (16–19). In these studies, p53-dependent apoptosis was not blocked by inhibition of transcription or translation, and p53 mutations have been defined whose behavior uncouples transcriptional activity from apoptotic activity within cells. In different cellular contexts, apoptosis and transcriptional activities of p53 appeared to be more tightly coupled (20, 21).

The measurement of cell-free apoptosis requires a biochemical surrogate for active apoptotic death, which has been facilitated by the discovery that apoptosis is executed by the activation of caspases. Caspases mediate apoptosis by cleaving a number of intracellular proteins (“death substrates”) (22, 23). One of the first identified caspase substrates is poly(ADP-ribose) polymerase (PARP), a target of caspase-3 (CPP32, apopain, YAMA) (24, 25). Because activation of this caspase is a common step in apoptosis, PARP cleavage by caspase-3 is now widely used as a biochemical marker for apoptosis, including in studies of cell-free systems (26–28).

Here we report that cell-free extracts from γ-irradiated, transformed p53 wild-type mouse or rat embryo fibroblasts induce caspase activation after a latent phase. This caspase activation requires functional p53: immunodepletion or inactivation of p53 either by recombinant SV40 large T antigen or anti-p53 IgG blocks caspase activation early, but not late, in cell-free incubations. Caspase activation is not blocked by immunodepletion of Bax, a transcriptional target of p53 (8), or cytochrome c, a co-activator of caspase-3 (27). Moreover an ATP analog, AMP-PNP, blocks p53-dependent caspase activation via cAMP/protein kinase A (PKA)-mediated repression, an effect which was corroborated in vivo by evidence that cAMP rescues cells from diverse triggers of p53-dependent apoptosis. These results demonstrate that p53 can transduce apoptotic signals through a Bax/cytochrome c-independent pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Fragmentation Analysis, and Extract Preparation—E1A/Ras-transformed mouse embryo fibroblasts (MEFs) were grown in Dulbecco's modified Eagle's medium containing 10% fetal

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1 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; MEF, mouse embryo fibroblast; REF, rat embryo fibroblast; PKA, protein kinase A; AMP-PNP, adenosine 5′-(β,γ-iminotriphosphate or adenosine 5′-(β,γ-iminotriphosphate); ATPγS, adenosine 5′-O-(thiotriphosphate).
bovine serum and 10% newborn calf serum. Primary rat embryo fibroblasts (REFs) and Myc/Ras-transformed REFs were maintained in minimal essential medium supplemented with 10% fetal bovine serum and 10% newborn calf serum. Exponentially growing cells at 60–80% confluence were either untreated or treated with 10 Gy of ionizing radiation using a Gammacell 40 equipped with a 137Cs source. At various times after irradiation, the cells were harvested, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline. For DNA fragmentation analysis, cells were harvested at 16 h after irradiation, and apoptotic DNA was isolated and analyzed as described (29).

To make cell-free extracts, the cell pellet was resuspended in 10 μl of a ice-cold buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 4 μg/ml Pefabloc, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). After incubating on ice for 15 min, the cells were disrupted by dunking 15 times in a Wheaton douncer with a tight pestle. The lysates were directly centrifuged at 100,000 × g for 1 h in a Sorvall RC Mi210 ultracentrifuge. The resulting supernatants (protein concentration ~8 mg/ml) were collected and stored at −80 °C.

**PARP Cleavage Assay—**For *in vitro* induction of PARP-cleaving activity, 8–10 μl of extracts were incubated at 32 °C for various times, after which 80 ng of recombinant PARP (a truncated form comprising the first 338 N-terminal amino acids and including the CPP32 cleavage site) was added for 5 min at 37 °C. For assays of *in vivo* activated PARP-cleaving caspases, 10 μl of extract was incubated with 80 ng of recombinant PARP for 15 min at 37 °C. At the end of the reaction, 4 × SDS sample buffer was added. The samples were boiled for 4 min, subjected to 12% SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The membrane was then probed with a monoclonal antibody (C2.10) against PARP (G. Poirier, Université Laval, Canada) and visualized by ECL (Amersham Pharmacia Biotech). Recombinant SV40 large T antigen, either as lysates (200–400 ng of total proteins) from recombinant T antigen baculovirus-infected Sf9 cells (generously provided by J. DeCaprio, Dana-Farber Cancer Institute) or as purified, bacterially produced T antigen (0.5–1 μg, Chimerex), was added to extracts at various time points during the 32 °C incubation period. Lysates from wild-type baculovirus-infected Sf9 cells or bovine serum albumin were used as controls. 200–400 ng of purified, polyclonal IgG against full-length p53 or the control IgG against the tyrosine kinase Fyn (both from Santa Cruz) was used in the indicated PARP cleavage assays. For cytochrome c experiments, 200 ng of cytochrome c (Sigma) was added to extracts at the beginning of the 32 °C incubation period. Nucleotides ATP, ATPγS, AMP-PNP, and cAMP were purchased from Boehringer Mannheim, and 1 mM final concentration was used in the indicated experiments. Catalytic subunit of PKA and a specific peptide inhibitor of PKA (PKI 6–22) were purchased from New England Biolabs and Calbiochem, respectively. 1 unit of PKA or 200 nm PKI 6–22 was used in the indicated experiments.

**Immunodepletion—**5 μg of monoclonal antibodies against mouse p53 (equal mixture of Ab-1, -3, -4, and -5; Oncogene Science), Bax (Trevigen), cytochrome c (Phar mingen), or microphthalmia (as a control) were incubated with 200 μl of extracts for 2 h on ice. The immune complexes were then cleared by two sequential rounds of incubation with a pre-coated and washed protein A/G-agarose bead pellet (from 30 μl of 50% suspension, Life Technologies, Inc.) for 2 h in a rotator at 4 °C. The beads were subsequently pelleted by centrifugation for 2 min in a microcentrifuge at 4 °C, and the resulting supernatants were collected and used for *in vitro* PARP cleavage assays.

**Flow Cytometry Analysis—**MyoC/Ras-transformed REFs were plated at 4 × 10⁵ per 60-mm plate. 8 h after plating, 10 μM forskolin in MeSO or the same volume of MeSO was added, and the cells were either untreated or immediately treated with 5 Gy of ionizing radiation. In serum starvation experiments, E1A/Ras-transformed p53 wild-type MEFs were plated at 4 × 10⁶ per 60-mm plate. 8 h after plating, the cells were washed with serum-free Dulbecco’s modified Eagle’s medium and maintained in Dulbecco’s modified Eagle’s medium containing either 20% or 2% serum (equal parts fetal bovine and newborn calf sera) with or without forskolin (10 μM). At 72 h after irradiation or 48 h after serum deprivation, adherent and floating cells were pooled, collected by centrifugation, and washed once with ice-cold phosphate-buffered saline. Staining with fluorescein isothiocyanate-annexin V or propidium iodide were performed according to the manufacturer’s instructions (Trevigen).

**RESULTS**

**Latent Caspase Activation in Extracts of Irradiated, Transformed Fibroblasts—**γ-irradiation triggers apoptosis in onco-gene-transformed MEFs and REFs, as indicated by the characteristic fragmentation of genomic DNA (Fig. 1a, lanes 3–6) (30). Although E1A/Ras-transformed p53 wild-type fibroblasts undergo radiation-induced apoptosis, genetically related E1A/Ras-transformed p53⁻/⁻ MEFs do not (lanes 1 and 2) (17, 30). Irradiated primary fibroblasts also fail to undergo apoptosis (lanes 7 and 8), instead undergoing cell cycle arrest (data not shown) (30). Consistent with the role of caspases in apoptosis, by 4 h after irradiation, extracts from transformed p53⁻/⁻ MEFs, but not from transformed p53⁻/⁻ MEFs, contain active caspases that readily cleave the death substrate PARP *in vitro* (exogenous truncated PARP, Fig. 1b).

In an effort to recapitulate the p53-dependent activation of caspases in a cell-free system, extracts were made from E1A/Ras-transformed p53⁻/⁻ MEFs within 1 h after irradiation. The extracts were then incubated at 32 °C for various times, after which recombinant PARP was added for 5 min, and PARP cleavage was determined by immunoblot. At 10 h, the extracts exhibited robust caspase activity (Fig. 2a). No PARP-cleaving activity was induced in extracts either from transformed p53⁻/⁻ MEFs, whether or not they had been irradiated or from unirradiated transformed p53⁻/⁻ MEFs as long as 24 h after incubating at 32 °C (Fig. 2b), suggesting that the caspase activity was specific for *in vivo* irradiation rather than *in vitro* incubation per se. Caspase induction with similar kinetics was also observed in extracts from irradiated Myc/Ras-transformed REFs (Fig. 2c). Consistent with the notion that irradiation...
leads to p53-driven cell cycle arrest (rather than apoptosis) in primary cells, extracts from untransformed REFs showed no caspase activity even after 24 h of incubation (Fig. 2d). This was not due to the absence of caspase precursors in the extracts because PARP-cleaving activity could be stimulated by addition of cytochrome c (Fig. 2d, lane 7), a co-activator of caspase-3 (27). The PARP-cleaving activity correlated with the appearance of the p20 subunit of activated caspase-3 (CPP32) (Fig. 2e).

Functional p53 Protein Is Required for Cell-free Caspase Activation—To determine whether activation of caspases in the cell-free extracts remains p53 dependent, we first performed immunodepletion experiments using monoclonal antibodies against p53. As shown in Fig. 3a, immunodepletion of p53 from extracts before the 32 °C incubation period completely abrogated the caspase activation (lane 3). Identical p53 immunodepletions at later time points (following 4 or 8 h at 32 °C) failed to block the caspase activation (lanes 4 and 5). No effect on the caspase activation was observed with control immunodepletions (lane 2). These results suggest that p53 function is required at an early stage upstream of caspase activation in the cell-free extracts. Addition of recombinant p53 derived from p53 baculovirus-infected S9 cells to extracts depleted of endogenous p53 has so far failed to activate caspases in this system (data not shown), which could reflect lack of proper post-translational modifications or other critical associations.

To further examine the p53 dependence of this caspase activation, we employed SV40 large T antigen, which has been demonstrated to block p53-mediated apoptosis in vivo (4). As shown in Fig. 3b, baculovirus lysates containing T antigen completely inhibited the induction of caspase activity in extracts from both irradiated transformed MEFs and REFs, but only when added at the beginning of the 32 °C incubation period (Fig. 3b, lanes 1, 2, and 7). Control lysates from wild-type baculovirus-infected S9 cells failed to do so (Fig. 3b, lanes 3 and 6). Purified bacterial recombinant T antigen also blocked the caspase activation in a dose-dependent fashion (Fig. 3b, 

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**FIG. 2.** *p53-dependent Apoptosis in Cell-free Extracts.* Extracts from irradiated E1A/Ras-transformed p53+/− MEFs (panels a and b, lanes 3 and 4) and p53−/− MEFs (panel b, lanes 1 and 2), Myc/Ras-transformed REFs (panels c and e), or untransformed REFs (panel d) were incubated at 32 °C for the indicated times, after which recombinant PARP was added for 5 min at 37 °C and PARP cleavage was monitored by immunoblot. In panel d, lane 7, 200 ng of cytochrome c was added at the beginning of the 32 °C incubation period. The blot in panel e was subsequently reprobed with a polyclonal antibody against the p20 subunit of activated CPP32 (caspase-3).

**FIG. 3.** *p53-dependent induction of PARP-cleaving activity in extracts from irradiated transformed p53+/− MEFs and REFs.* p53 was depleted from the MEF extracts with p53-specific monoclonal antibodies at the indicated time points before (0) or during (4 or 8 h) the 12-h 32 °C incubation period (a). Control immunodepletion (Ctrl) was performed with a monoclonal antibody against microphthalmia at the beginning of the 32 °C incubation period. Lysates from recombinant T antigen baculovirus-infected S9 cells were added to the extracts at either the beginning (E for early) or the end (L for late) of the 12-h 32 °C incubation period (b). For the control, lysates from wild-type baculovirus-infected S9 cells were added at the beginning (E) of the 32 °C incubation period. Lanes 8–10, purified bovine serum albumin (1 mg) or bacterially produced T antigen (rTAG, 0.5–1 μg) was added at the beginning of the 32 °C incubation period. Purified, polyclonal IgG against full-length p53 or polyclonal IgG against the tyrosine kinase Fyn (Ctrl) was added to the extracts (MEF, lanes 1–3; REF, lanes 4–7) at either the beginning (E) or the end (L) of the 12-h 32 °C incubation period.
is indicated (a). The presence of T antigen (lanes 1–3) at the beginning of the 12-h 32 °C incubation period, followed by the PARP cleavage assay (a). A small amount of residual anti-p53 antibody heavy chain sometimes seen after immunodepletion is indicated (IgH). Extracts from irradiated p53−/− MEFs were incubated with anti-p53, anti-Bax, anti-cytochrome c, or anti-microphthalmia (Ctrl) monoclonal antibodies, and the resulting immune complexes were cleared using protein A/G-agarose beads (b). The resulting extracts were then used for PARP cleavage assays. The immunoblot was sequentially reprobed with antibodies against p53, Bax, and cytochrome c.

lanes 8–10). Similarly, purified polyclonal IgG against full-length p53, but not nonspecific purified polyclonal IgG, prevented the induction of PARP-cleaving activity in both MEF and REF extracts only when added before the 32 °C incubation period (Fig. 3c).

p53 and Cytochrome c Function in Distinguishable Apoptotic Pathways in the Cell-free Extracts—Failure of the extracts to activate PARP suggests that cytochrome c is not linearly downstream of p53 in this apoptosis pathway. In fact, subcellular fractionation in the presence of sucrose shows that cytochrome c is not cytosolic 1 h after irradiation, but only emerges during extract preparation (data not shown), probably as a result of disrupting mitochondrial integrity by hypotonic lysis. Therefore, extracts prepared from both p53−/− and p53+/+ cells with or without irradiation contained similar levels of released cytochrome c (data not shown), which was not capable of inducing caspases, although high levels of exogenous cytochrome c can activate caspases in these extracts.

Fig. 4. Bax and cytochrome c are not required for the cell-free p53-dependent caspase activation. Cytochrome c was added to the MEF extracts depleted of p53 (lanes 4 and 5) or in the presence of T antigen (lanes 1–3) at the beginning of the 12-h 32 °C incubation period, followed by the PARP cleavage assay (a). A small amount of residual anti-p53 antibody heavy chain sometimes seen after immunodepletion is indicated (IgH). Extracts from irradiated p53−/− MEFs were incubated with anti-p53, anti-Bax, anti-cytochrome c, or anti-microphthalmia (Ctrl) monoclonal antibodies, and the resulting immune complexes were cleared using protein A/G-agarose beads (b). The resulting extracts were then used for PARP cleavage assays. The immunoblot was sequentially reprobed with antibodies against p53, Bax, and cytochrome c.

egans CED-4, Apaf-1, and caspase-9 (31, 32). Furthermore, the ability of cytochrome c to induce PARP-cleaving activity in extracts depleted of p53 or in the presence of T antigen suggested that cytochrome c may function either downstream of p53 or in a distinct pathway to activate caspases. As shown in Fig. 4b, in contrast to immunodepletion of p53 which blocked caspase activation, extracts depleted of either Bax or cytochrome c remained fully active for PARP cleavage after the 32 °C incubation period. The failure of Bax depletion to prevent caspase activation in the cell-free system is consistent with the report that p53-dependent apoptosis remains functional in Bax−/− mouse thymocytes (33), although it could play a role in setting cellular apoptosis thresholds because measurable effects have been detected in Bax-deficient fibroblasts (34). The finding that cytochrome c depletion did not block the ability of the extracts to activate caspases suggests that cytochrome c is not linearly downstream of p53 in this apoptosis pathway. In fact, subcellular fractionation in the presence of sucrose shows that cytochrome c is not cytosolic 1 h after irradiation, but only emerges during extract preparation (data not shown), probably as a result of disrupting mitochondrial integrity by hypotonic lysis. Therefore, extracts prepared from both p53−/− and p53+/+ cells with or without irradiation contained similar levels of released cytochrome c (data not shown), which was not capable of inducing caspases, although high levels of exogenous cytochrome c can activate caspases in these extracts.

Protein Kinase A Blocks p53-dependent Apoptosis in Vitro and In Vivo—The activation of caspases in this cell-free system is unlikely to require hydrolysis of ATP, whose addition even partially inhibited induction of PARP-cleaving activity (Fig. 5a, lane 2). The nonhydrolyzable ATP analog, ATPγS, had minimal effect on caspase activation (Fig. 5a, lane 3). However, another nonhydrolyzable ATP analog, AMP-PNP, completely abrogated the activation of caspases (Fig. 5a, lane 4). This inhibitory effect was observed when AMP-PNP was added at the beginning but not later in the 32 °C incubation period (Fig. 5a, lanes 5–7), suggesting that AMP-PNP inhibited an activity upstream of caspases.

Interestingly, both ATP and AMP-PNP, but not ATPγS, can be converted to cAMP (35), which in turn can activate PKA. Three lines of evidence suggested essential roles for cAMP and PKA in the AMP-PNP-mediated inhibition of caspase activation. First, a purified specific peptide inhibitor of PKA (PKI) strongly relieved the inhibitory effect of AMP-PNP (Fig. 5b, lanes 1–3). Second, when added to the cell-free extracts at the beginning of the 32 °C incubation period, cAMP inhibited the caspase activation as potently as AMP-PNP (Fig. 5b, lane 4–6). Finally, purified PKA catalytic subunit completely abolished the induction of caspase activity in the presence of ATP, but not its nonhydrolyzable analog ATPγS (Fig. 5b, lanes 7–10), and this inhibition was reversed by the specific PKA peptide inhibitor. Thus cAMP/PKA appear to inhibit the p53-dependent apoptosis reflected in these extracts.

Finally, the relevance of these in vitro cAMP effects was examined in cell-based apoptosis studies. The cAMP-inducing agent forskolin was examined for its effects on p53-dependent apoptosis in vivo. As shown in Fig. 5c, forskolin significantly protected radiation-induced apoptosis in transformed fibroblasts. In addition, the apoptotic response to serum starvation was significantly blunted by forskolin (Fig. 5c). Of note, the activation of PKA by forskolin is short-lived (because of homeostatic down-regulation, see Ref. 36) and may therefore underestimate the magnitude of the protection because of this experimental system. Nonetheless, these results demonstrate that the cell-free apoptosis assay described here predicts effects that are in agreement with in vivo behavior, and that the
AMP-PNP was added at the end of the 32 °C incubation period, followed by PARP cleavage assays. (a) AMP-PNP inhibits the in vitro induction of PARP-cleaving activity. 1 mM ATP, ATPγS, or AMP-PNP was added to MEF extracts at the beginning of the 32 °C incubation period, or in lane 7, AMP-PNP was added at the end of the 32 °C incubation period, followed by PARP cleavage assays. Panel b, inhibition of the caspase activation by AMP-PNP is mediated by PKA. Indicated nucleotides (1 mM), the catalytic subunit of PKA (1 unit), or a specific PKA inhibitor (PKI, 200 nM) were added to the MEF extracts at the beginning of the 12-h 32 °C incubation period, followed by PARP cleavage assays. Panel c, activation of PKA by forskolin protects oncogene-transformed cells from apoptosis induced either by irradiation (Myc/Ras REFs) or by serum starvation (E1A/Ras MEFs). Apoptosis was quantitated by fluorescein isothiocyanate-annexin V staining at 72 h post-irradiation or by propidium iodide staining at 48 h post-serum deprivation. The bars represent averages with indicated S.E. for the experimental groups (containing three or more samples).

FIG. 5. Panel a, AMP-PNP inhibits the in vitro induction of PARP-cleaving activity. 1 mM ATP, ATPγS, or AMP-PNP was added to MEF extracts at the beginning of the 32 °C incubation period, or in lane 7, AMP-PNP was added at the end of the 32 °C incubation period, followed by PARP cleavage assays. Panel b, inhibition of the caspase activation by AMP-PNP is mediated by PKA. Indicated nucleotides (1 mM), the catalytic subunit of PKA (1 unit), or a specific PKA inhibitor (PKI, 200 nM) were added to the MEF extracts at the beginning of the 12-h 32 °C incubation period, followed by PARP cleavage assays. Panel c, activation of PKA by forskolin protects oncogene-transformed cells from apoptosis induced either by irradiation (Myc/Ras REFs) or by serum starvation (E1A/Ras MEFs). Apoptosis was quantitated by fluorescein isothiocyanate-annexin V staining at 72 h post-irradiation or by propidium iodide staining at 48 h post-serum deprivation. The bars represent averages with indicated S.E. for the experimental groups (containing three or more samples).

CAMP/PKA pathway can negatively regulate p53-dependent apoptosis.

**DISCUSSION**

This report demonstrates p53-mediated apoptosis in a cell-free system. Significantly, the p53-dependent activation of caspases in this system does not require the presence of Bax or cytochrome c. These findings suggest that a distinct set of factors relay the p53-dependent apoptotic signals to caspases. A recent study has shown that Bax may act upstream of cytochrome c; overexpression of Bax induces the release of cytochrome c, which in turn activates caspase-3 and results in cell death (37). Cytochrome c may well be an essential component in Bax-dependent apoptosis. According to the death cycle model (38, 39), it is also possible that within cells cytochrome c could play a role in caspase activation through a Bax-independent pathway in which p53-mediated activation of caspases promotes the release of cytochrome c, which in turn amplifies the apoptotic cascade. This hypothesis is also consistent with the report that there is synergy between transcription-dependent and -independent functions of p53 in apoptosis (40).

One reproducible feature of the system described here is the relatively long delay (8–12 h) before in vitro induction of caspase activity. There are several important potential reasons for these kinetics. First, caspase activation requires approximately 4 h even within intact cells, thereby representing a relatively slow cascade (or multistep process) _in vitro_. Second, this assay specifically employs 32 °C incubation periods which, although kinetically suboptimal, diminishes nonspecific degradation of exogenous PARP. Third, components within the extracts are significantly diluted relative to intracellular conditions. Finally, the _in vitro_ latent period before caspase activation was found to shorten with increasing _in vitro_ incubation after irradiation, eventually passing the p53-dependent stage (data not shown). The same is true for other stimuli of apoptosis such as growth factor deprivation, which in the extreme gives “spontaneous” caspase activation upon extract preparation. Thus for this system the viability of starting cell populations is crucial for the ability to discriminate the slower _in vitro_ p53-dependent activity.

The tumor cells used here have been transformed by two oncogenes, Ras plus either E1A or Myc. Therefore, the precise contribution of each oncogene to the apoptotic pathway is uncertain at this time. Stable overexpression of Myc alone in REFs has been shown to produce immortalization without transformation (as measured by solid tumor formation) (41), but the apoptotic propensity of these cells has not been reported. In contrast, p53−/− cells transiently and forcibly overexpressing E1A readily undergo apoptosis, even to a degree that limits generation of stable lines (42). It would be of interest to examine any such immortalized cells for the apoptotic activity reported here as they could provide clues to the mechanistic connection between oncogene activity and p53-dependent apoptosis.

In this tumor cell system, PKA blocks p53-dependent caspase activation in cell-free extracts and protects transformed embryonic fibroblasts from apoptosis triggered by γ-irradiation or by serum deprivation. Activation of PKA has been reported to either inhibit or promote apoptosis depending on experimental systems (43). The ability of PKA to inhibit the caspase activation _in vitro_ suggests that it functions by phosphorylation of a protein(s) present in the extracts, which is consistent with a report that cAMP prevents apoptosis by translation- and transcription-independent mechanisms (44). p53 itself has been shown to serve as a PKA substrate (38, 39), but it is unclear whether this correlates with its apoptotic activity. Identification of the PKA target(s) may reveal important components of this pathway. Finally, recognition that elevated cAMP may repress p53-dependent apoptosis carries clinical implications for patients with p53 wild-type malignancies undergoing curative antineoplastic therapy, because numerous common agents (for example, caffeine) regulate cAMP metabolism.

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