DNA damage-induced apoptosis requires the DNA-dependent protein kinase, and is mediated by the latent population of p53

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Mouse embryo fibroblasts (MEFs) expressing the adenovirus E1A protein undergo apoptosis upon exposure to ionizing radiation. We show here that immediately following γ -irradiation, latent p53 formed a complex with the catalytic subunit of the DNA-dependent protein kinase (DNA-PK_{CS}). The complex formation was DNase sensitive, suggesting that the proteins came together on the DNA, conceivably at strand breaks. This association was accompanied by phosphorylation of pre-existing, latent p53 at Ser18 (corresponding to Ser15 in human p53), which was not found in DNA-PK_{CS}^{-/-} cells. Most significantly, DNA damage-induced apoptosis was abolished in both DNA-PK_{CS}^{-/-} and p53^{-/-} cells. In addition, blocking synthesis of inducible p53 by cycloheximide did not abrogate apoptosis, suggesting that the latent population of p53 is sufficient for executing the apoptotic program. Finally, E1A-expressing MEFs from a p53 ‘knock-in’ mouse where Ser18 was mutated to an alanine had an attenuated apoptotic response, indicating that phosphorylation of this site by DNA-PK is a contributing factor for apoptosis.

Keywords: apoptosis/DNA damage/DNA-dependent protein kinase/p53/Ser15

Introduction

p53 can integrate signals from the intercellular and intracellular environment to respond to a variety of genotoxic and non-genotoxic stimuli. In unstressed cells, wild-type p53 has a very short half-life (20–30 min), existing in a latent form (Hupp *et al.*, 1995; reviewed in Giaccia and Kastan, 1998; Oren, 1999). Exposure of cells to genotoxic agents leads to two independent events. The first is induction and stabilization of p53 protein levels, probably involving phosphorylation of N-terminal serines, and destabilization of the negative-regulatory mdm2 interaction (Shieh *et al.*, 1997; Siliciano *et al.*, 1997; Chehab *et al.*, 1999; Shieh *et al.*, 1999; Unger *et al.*, 1999a; Sakaguchi *et al.*, 2000). The second event that occurs following genotoxic stress is the activation of p53,

leading to cell cycle arrest or apoptosis. While both events can be considered essential, it is clear that they are independent and separable, because stabilization of p53 using the calpain/proteasome inhibitor, LLnL (Maki *et al.*, 1996; Shieh *et al.*, 1997), or via oncogene expression (Debbas and White, 1993; Lowe and Ruley, 1993; Lowe *et al.*, 1993) is insufficient for activation of p53-dependent cell-cycle arrest or apoptosis.

Given the critical nature of this protein, it is likely that multiple upstream molecular pathways will have evolved leading to either the induction and stabilization of p53, or a conversion of p53 from an inactive to an activated form. The alternate mechanisms may be invoked for different cell types or different physiological stimuli (reviewed in Giaccia and Kastan, 1998). For example, the protein product encoded by the *Atm* gene mutated in ataxia telangiectasia (AT) has been shown to be dispensable for p53-dependent apoptosis in thymus tissue, in thymocytes and in the choroid plexus epithelium of a transgenic mouse brain tumor model (Barlow *et al.*, 1997; Liao *et al.*, 1999; Wang *et al.*, 2000). However, others have shown that *Atm* is required for p53-dependent apoptosis of neurons in the developing central nervous system (Herzog *et al.*, 1998). Other reports suggest a partial reduction in p53-dependent apoptosis of *Atm*^{-/-} thymus cells (Xu and Baltimore, 1996) or thymocytes (Westphal *et al.*, 1997). Therefore, the requirement for *Atm* in p53-dependent apoptosis remains tenuous.

Nevertheless, it has been reasonably demonstrated that *Atm* is involved in the pathway leading to stabilization of p53 following genotoxic stress. However, strictly from a mechanistic viewpoint, it has never been shown whether ‘unstable’ p53 in AT cells can be activated for cell cycle arrest. It is conceivable that a pathway, independent of stabilization, leads to the activation of p53; therefore, despite the low level of unstable p53 in AT cells, p53 may still be active. This may explain why AT cells have a subdued and delayed p53 response to ionizing radiation (Kastan *et al.*, 1992; Lu and Lane, 1993).

Another good candidate for detecting and relaying the DNA damage signal to p53 is the DNA-dependent protein kinase (DNA-PK). It is a nuclear serine/threonine kinase that requires DNA double-strand breaks (Gottlieb and Jackson, 1993) or single-strand nicks or gaps (Morozov *et al.*, 1994) in the DNA for its activity. DNA-PK is involved in DNA double-strand break repair and V(D)J recombination. The kinase consists of a 470 kDa catalytic subunit, DNA-PK_{CS}, and a DNA binding regulatory component that consists of the Ku70/Ku80 heterodimer (Gottlieb and Jackson, 1993). Defects in DNA-PK_{CS} or Ku result in hypersensitivity to ionizing radiation, defects in DNA double-strand break repair (Getts *et al.*, 1994; Taccioli *et al.*, 1994; Lees-Miller *et al.*, 1995), severe

Fig. 1. DNA-PK is required for oncogene-sensitized, p53-dependent apoptosis. (A) MEFs expressing E1A were grown on glass cover slips. Cells were either untreated, or were γ -irradiated (IR). Twenty-four hours after irradiation, the cells were fixed, stained with DAPI and analyzed for morphological characteristics associated with apoptosis. (B) Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) and represent the average of three independent experiments $\pm$ SEM.

immunodeficiency (Kurimasa *et al.*, 1999) and cancer predisposition (Jhappan *et al.*, 1997).

Like Atm, there are conflicting reports about the role of DNA-PK in p53-dependent pathways. We developed an *in vitro* translation/DNA-binding assay to study p53 (Woo *et al.*, 1998). This assay system allowed for the analysis of the activation pathways for p53 independent of stabilization, since p53 is translated at sufficiently high levels to not require further stabilization. These studies demonstrated that DNA-PK, in synergy with another factor, could activate p53. Since our report, several groups have developed the DNA-PK^{CS} null transgenic mouse. These reports have shown that DNA-PK^{CS} is not required for stabilization of p53, nor is it involved in p53-dependent cell-cycle arrest in response to genotoxic stress (Burma *et al.*, 1999; Jimenez *et al.*, 1999; Jhappan *et al.*, 2000). However, the entire scope of p53-dependent responses was not analyzed. It has since been shown that DNA-PK^{CS}^{-/-} mice have complete abrogation of p53-dependent apoptosis in the thymus (Wang *et al.*, 2000).

DNA-PK^{CS} null transgenic mice have defects in thymocyte development, making it difficult to study thymocyte apoptosis. Therefore, we analyze mouse embryo fibroblasts (MEFs) expressing the adenovirus E1A oncoprotein to further dissect apoptotic mechanisms. The MEF model allowed for comparison of cells undergoing cell cycle arrest versus apoptosis in response to DNA damage. In response to genotoxic stress, wild-type MEFs would undergo a p53-dependent cell cycle arrest at

G₁ phase of the cell cycle (Kastan *et al.*, 1992). In contrast, growth-deregulated MEFs, such as those expressing E1A, E2F or Myc, become sensitized to p53-dependent apoptosis in response to DNA damage (Debbas and White, 1993; Lowe and Ruley, 1993; Lowe *et al.*, 1993; Hermeking and Eick, 1994; Qin *et al.*, 1994; Shan *et al.*, 1994; Wagner *et al.*, 1994; Wu and Levine, 1994). These oncogenes promote S-phase entry, suggesting that G₁ phase of the cell cycle is incompatible with initiation of apoptosis. In this context, G₁ arrest can be considered anti-apoptotic.

Using this approach, we have demonstrated that DNA-damage-induced apoptosis in MEFs expressing E1A is abolished in both DNA-PK^{CS}^{-/-} and p53^{-/-} cells. Also, DNA-PK^{CS} specifically associates with the pre-existing 'latent' population of p53 in E1A-expressing MEFs immediately following DNA damage, but not in MEFs without E1A. In addition, DNA-PK is required for phosphorylation of the same 'latent' population of p53 at Ser18 (from here on referred to as the human p53 equivalent, Ser15) in response to γ -irradiation. Furthermore, blocking synthesis of 'inducible' p53 following γ -irradiation did not abrogate the apoptotic program, indicating that the pre-existing 'latent' population of p53 is sufficient for executing the apoptotic program. Finally, E1A-expressing MEFs from a p53 'knock-in' mouse where Ser15 is mutated to an alanine, had an attenuated apoptotic response, indicating that phosphorylation of this site by DNA-PK is a critical step in the apoptotic program.

Results

DNA-PK is required for p53-dependent apoptosis

The adenovirus E1A oncogene sensitizes primary fibroblasts to apoptosis induced by DNA damage (Debbas and White, 1993; Lowe *et al.*, 1993). Here, the 12S E1A gene was introduced into MEFs by retroviral-mediated gene transfer. Clonal isolates of wild-type, p53^{-/-} and DNA-PK^{CS}^{-/-} MEFs expressing E1A were obtained. E1A expression was verified by western blotting and anti-E1A immunofluorescent staining (data not shown). These cells were grown on glass cover slips, and irradiated with 5 Gy ionizing radiation to induce p53-dependent apoptosis. Apoptosis was monitored by staining the cells with 4',6'-diamidino-2-phenylindole (DAPI) followed by fluorescence microscopy to visualize the morphological characteristics associated with apoptotic cells, including nuclear breakdown and heterochromatin aggregation (Hendzel *et al.*, 1998).

As expected, untreated cells of each genotype did not have any of the morphological characteristics of an apoptotic cell (Figure 1A). In agreement with previous results, γ -irradiating wild-type MEFs expressing E1A led to an average of 39% of cells with apoptotic morphology (Figure 1). As expected, p53^{-/-} cells had a severely attenuated apoptotic response, confirming p53-dependency for this program. DNA-PK^{CS}^{-/-} cells, like the p53^{-/-} cells, were resistant to DNA-damage-induced apoptosis. Therefore, oncogene-associated apoptosis requires both p53 and DNA-PK. These results are in agreement with the observation that both p53 and DNA-PK are required for apoptosis in the mouse thymus (Wang *et al.*, 2000). It is

Fig. 2. DNA-PK–p53 complex formation in apoptotic cells. (A) p53 co-immunoprecipitates with DNA-PK. MEFs or MEFs expressing E1A were either untreated (CTRL) or γ -irradiated (IR). Cell extracts were then prepared and DNA-PK was immunoprecipitated from the cell extracts using the anti-DNA-PK_{CS} antibody C-19 (Santa Cruz), followed by SDS–PAGE and transfer to nitrocellulose. The membrane was probed for p53 using the anti-p53 polyclonal antibodies, FL393 (Santa Cruz). Total p53 and total DNA-PK_{CS} protein levels in the cell extracts are also shown. [To make the input p53 protein level comparable, twice as much cell extract (100 μ g of protein) was used from the MEFs versus the MEFs expressing E1A.] (B) DNA-PK_{CS}–p53 complex formation is DNA dependent. (i) Cell extracts from untreated (CTRL) or γ -irradiated (IR) wild-type MEFs expressing E1A were immunoprecipitated with the anti-DNA-PK_{CS} antibody C-19 (Santa Cruz), in the absence of any pre-treatment, (ii) pre-treating the cell extracts with ethidium bromide (EtBr) prior to the IP, or (iii) pre-treating the cell extracts with DNase prior to the IP. Following SDS–PAGE and transfer to nitrocellulose, the membrane was probed with the anti-p53 antibody, FL-393 (Santa Cruz). (iv) Total p53 protein levels in the cell extracts.

noteworthy that MEFs (expressing E1A) derived from DNA-PK_{CS}^{+/–} littermates produced a similar level of apoptosis to the wild-type cells (data not shown) indicating that there is no gene-dosage effect on this pathway. In addition, uninfected MEFs of all genotypes did not have any of the morphological characteristics of apoptotic cells following γ -irradiation (data not shown) verifying that E1A was required for sensitizing these cells to apoptosis.

DNA-PK interacts with p53

As DNA breaks are known to activate DNA-PK *in vivo* (Lees-Miller *et al.*, 1990; Gottlieb and Jackson, 1993; Morozov *et al.*, 1994), and DNA-PK is capable of phosphorylating Ser15 and Ser37 of human p53 *in vitro* (Lees-Miller *et al.*, 1990, 1992), it is reasonable to suggest that DNA-PK may be an upstream regulator of p53 function in response to DNA damage. Interestingly, p53 can bind to DNA ends, excision-repair damage sites or internal deletion loops (Lee *et al.*, 1995). In view of that, it is possible for both p53 and DNA-PK to be localized at the site of DNA damage and repair, where phosphorylation or other activating signals can be processed. Indeed, p53 is

phosphorylated more efficiently by DNA-PK when both proteins are bound to neighboring sites on the same piece of DNA (Lees-Miller *et al.*, 1992).

In light of these observations, it was of interest to investigate a possible interaction between these two proteins. Uninfected wild-type MEFs, or wild-type MEFs expressing E1A were irradiated with 5 Gy of ionizing radiation. DNA-PK_{CS} from cell extracts were immunoprecipitated with an anti-DNA-PK antibody and then immunoblotted for p53. It is reported here for the first time that p53 co-immunoprecipitates with DNA-PK_{CS} in cell extracts from irradiated MEFs with E1A, but not in cell extracts of uninfected MEFs (Figure 2A). This is important because uninfected MEFs respond to irradiation by arresting at G₁ phase of the cell cycle, whereas MEFs expressing E1A undergo apoptosis. In view of the dual requirement of p53 and DNA-PK_{CS} for an ionizing radiation (IR)-induced apoptotic response in the E1A-expressing cells, it seems logical to speculate that the DNA-PK_{CS}–p53 complex occurs only in apoptotic cells.

DNA-PK_{CS}–p53 interaction is sensitive to DNA disrupting agents

As p53 and the DNA-PK holoenzyme can bind non-specifically to DNA, it is possible for both proteins to be localized to the site of DNA damage and repair, where phosphorylation or other activating signals can be processed. Indeed, p53 is phosphorylated more efficiently by DNA-PK when both proteins are bound to neighboring sites on the same piece of DNA (Lees-Miller *et al.*, 1992). To test this possibility, cell extracts were pre-treated with ethidium bromide to determine whether this could disrupt DNA-PK_{CS}–p53 complex formation. As before, p53 co-immunoprecipitated with DNA-PK_{CS} from cell extracts of irradiated cells (Figure 2Bi). However, when the same cell extract was pre-treated with ethidium bromide, the co-immunoprecipitation (co-IP) was virtually eliminated (Figure 2Bii).

As DNA-PK is activated by DNA, the use of ethidium bromide to disrupt non-specific DNA–protein interactions by unwinding the DNA helix may have had the inadvertent effect of inactivating DNA-PK, and it is this effect that may have impeded the co-IP with p53. Therefore, the cell extracts were instead pre-treated with the endonuclease DNase I, which would not intercalate and unwind the DNA, but rather degrade exposed DNA, potentially breaking the tether between two DNA-bound proteins. Under these conditions, the DNA-PK_{CS}–p53 co-IP was also blocked, similar to the effect of ethidium bromide (Figure 2Biii). Total p53 protein levels are shown in Figure 2Biv.

Although a cross-linking agent was not used in these experiments, the DNA-PK_{CS}–p53 co-IP was initially discovered using the cross-linking agent dithiobis-succinimidyl propionate (DSP; data not shown). This cross-linker has a 12 Å spacer arm indicating that these two proteins are not non-specifically linked together by DNA, but rather, they are very closely associated.

DNA-PK interacts with latent p53 in response to DNA damage

DNA-PK-deficient cells are radiosensitive and are defective for DNA double strand break repair; in DNA-PK

Fig. 3. DNA-PK_{CS} interacts with latent p53 in response to γ -irradiation. (A) DNA-PK–p53 complex formation is an immediate-early response to DNA damage. Cell extracts from untreated (CTRL) or γ -irradiated (IR) MEFs expressing E1A were immunoprecipitated with the anti-DNA-PK_{CS} antibody C-19. The γ -irradiated cells were harvested at different time intervals post IR (note, the 0 h time-point represents a sample that had been irradiated, and then processed immediately). Following SDS–PAGE and transfer to nitrocellulose, the membrane was probed with the anti-p53 antibody, FL393. Total p53 protein levels in the cell extracts are also shown. (B) Effect of cycloheximide on total p53 levels in IR-treated MEFs expressing E1A. Cells were pre-treated with cycloheximide (CHX; 30 μ g/ml) for 15 min prior to exposure to γ -irradiation. Cells were harvested at different time intervals post-IR. Total p53 protein levels as detected by western blot are shown. (C) Formation of IR-induced DNA-PK–p53 complex in the absence of protein synthesis. Extracts from cells left untreated (CTRL), cycloheximide-treated, γ -irradiated (followed by 60 min recovery) or cells pre-treated with cycloheximide for 15 min followed by IR (60 min recovery post-IR) were immunoprecipitated with the anti-DNA-PK_{CS} antibody C-19. Following SDS–PAGE and transfer to nitrocellulose, the membrane was probed with the anti-p53 antibody, FL393.

proficient cells, DNA double strand breaks are repaired within 30 min following exposure to IR (Lees-Miller *et al.*, 1995). To assess the timing of the DNA-PK–p53 interaction, cells were harvested at different intervals following exposure to IR. As before, p53 did not co-immunoprecipitate with DNA-PK_{CS} in unirradiated MEFs expressing E1A (Figure 3A, lane 1). Following exposure to 5 Gy ionizing radiation, p53 co-immunoprecipitated with DNA-PK_{CS} almost immediately; this association decreased with time to almost undetectable level after 2.5–3 h (Figure 3A, lanes 2–8).

That DNA-PK interacts with p53 immediately following exposure to γ -irradiation suggests that it is the pre-existing latent form of p53, and not newly synthesized radiation-induced p53, that complexes with DNA-PK. To test this assertion, cells were pre-treated with the protein synthesis inhibitor cycloheximide prior to exposure to γ -irradiation. In the absence of cycloheximide, p53 protein levels gradually increase with time following exposure to IR (Figure 3B, lanes 1–5). Pre-treating the cells with

Fig. 4. DNA-PK phosphorylates latent p53 at Ser15 in response to γ -irradiation. (a and b) MEFs (expressing E1A) derived from either DNA-PK^{+/+} or DNA-PK^{-/-} mice were analyzed. Cells were untreated (CTRL), γ -irradiated (IR), treated with cycloheximide (CHX; 30 μ g/ml), or treated with both IR and cycloheximide. The cells were harvested at different time intervals following the respective treatments (0, 0.5, 1 and 1.5 h post-treatment). Following SDS–PAGE and transfer to nitrocellulose, the membrane was probed with an anti-phosphoserine-15 antibody. (c and d) The same cell extracts used in (a) and (b) was analyzed for total p53 protein levels by western blotting.

cycloheximide prevented induction and accumulation of p53 following IR (Figure 3B, lanes 6–10). Therefore, the population of p53 in these samples must only be the pre-existing latent form, and demonstrates the efficacy of cycloheximide treatment. The amount of p53 that co-immunoprecipitated with DNA-PK_{CS} in the absence of cycloheximide is shown in Figure 3C (lane 3). A similar amount of p53 co-immunoprecipitated with DNA-PK_{CS} even with cycloheximide treatment (Figure 3C, lane 4), indicating that DNA-PK_{CS} interacts with the pre-existing latent population of p53 in response to γ -irradiation.

DNA-PK is required to phosphorylate latent p53 in response to IR

Several kinases, including DNA-PK, have been shown to phosphorylate p53 at Ser15 *in vitro*. This is believed to be significant because DNA damage induces phosphorylation of p53 at Ser15 *in vivo* (Shieh *et al.*, 1997; Siliciano *et al.*, 1997). Therefore, it was important to determine whether DNA-PK is required for phosphorylation of p53 at Ser15 in response to DNA damage. For this, DNA-PK_{CS}^{+/+} cells or DNA-PK_{CS}^{-/-} cells were analyzed (Kurimasa *et al.*, 1999). Cell extracts were immunoblotted with an anti-phosphoserine-15 antibody. p53 was not phosphorylated in untreated DNA-PK_{CS}^{+/+} or DNA-PK_{CS}^{-/-} cells (Figure 4a, lane 1, and b, lane 1, respectively). Immediately following γ -irradiation, the heterozygous cells had detectable Ser15 phosphorylation, which lasted for at least 1.5 h (i.e. the duration of the experiment; Figure 4a, lanes 2–5). The DNA-PK_{CS} null cells had significantly less Ser15 phosphorylation immediately following γ -irradiation, but this had increased to match phosphoserine-15 levels in the heterozygous cells by the 1.5 h time point (Figure 4b, lanes 2–5).

The lack of immediate phosphorylation of p53 in the irradiated DNA-PK_{CS} null cells suggests that DNA-PK is involved in phosphorylation of the latent population of p53. The ability of DNA-PK to complex with latent p53 in response to γ -irradiation would be consistent with this notion. Again, using cycloheximide to prevent induction and accumulation of p53 following IR allowed for analysis of phosphorylation of latent p53. Cycloheximide, on its

own, did not induce Ser15 phosphorylation in either DNA-PK_{CS} heterozygous or null cells (Figure 4a, lanes 6–9, and b, lanes 6–9, respectively). Pre-treating the heterozygous cells with cycloheximide, followed by γ -irradiation resulted in immediate phosphorylation of latent p53 at Ser15 (Figure 4a, lanes 10–13). Under the same treatment protocol, the DNA-PK_{CS} null cells did not have any detectable phosphorylation of latent p53 at Ser15 (Figure 4b, lanes 10–13). Total p53 protein levels were comparable in both the DNA-PK_{CS} heterozygous cells and the DNA-PK_{CS} null cells (Figure 4c and d, respectively), thus excluding the possibility that differences in phosphorylation of p53 at Ser15 are caused by differences in p53 protein levels.

This experiment therefore shows that DNA-PK_{CS} is responsible for phosphorylation of latent p53 at Ser15. It is noteworthy that p53 synthesized after IR treatment ('inducible p53') is also phosphorylated at Ser15 (Figure 4b, lane 5). This is probably the Atm-dependent Ser15 phosphorylation described previously (Banin *et al.*, 1998; Canman *et al.*, 1998; Jimenez *et al.*, 1999), since it occurs even in DNA-PK^{-/-} cells.

The pre-existing, latent population of p53 is sufficient for executing the apoptotic program

Since DNA-PK can only interact with and phosphorylate latent p53, it was important to determine whether this subpopulation of p53 would be sufficient to carry out the apoptotic program. Pre-treating cells with cycloheximide prevented induction and accumulation of p53 following IR (see Figure 3B). Therefore, apoptosis could be evaluated in the presence of cycloheximide to determine whether newly synthesized, DNA-damage-induced p53 was required for apoptosis. As prolonged cycloheximide treatment would eventually cause necrotic cell death (after ~24 h), an early apoptotic marker had to be analyzed. In normal viable cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organization of phospholipids lead to exposure of PS on the cell surface. PS translocation to the cell surface precedes nuclear breakdown, and chromatin condensation. Externalized PS can be detected through interaction with annexin V (Fadok *et al.*, 1992).

To this end, MEFs expressing E1A were mock-treated, γ -irradiated (IR), treated with cycloheximide or treated with both cycloheximide and IR. This was followed by staining the cells with calcein AM and Cy3-conjugated annexin V (Figure 5). Calcein AM is non-fluorescent unless hydrolyzed in viable cells to become a fluorescent fluorescein derivative. A viable cell that stains with annexin V is in the early stages of apoptosis, whereas a non-viable cell that is annexin V-positive can be either late apoptotic or necrotic. Similar to the morphological apoptotic assay, wild-type MEFs with E1A underwent apoptosis in response to IR as shown by dual-positive staining. Treating these cells with cycloheximide alone did not induce apoptosis. Pre-treating the cells with cycloheximide followed by γ -irradiation did not significantly reduce the level of apoptosis. Therefore, the pre-existing latent population of p53 is sufficient to carry out the apoptotic program, and DNA damage-induced apoptosis does not require inducible p53. It should be noted that in

this assay, DAPI staining was used only as a counterstain to visualize how many cells were present. Apoptotic morphology was not observed because the assay was carried out only 5 h after exposure to IR, when nuclear blebbing and heterochromatin aggregation had yet to occur.

Phosphorylation of p53 at Ser15 is important for programmed cell death

In four separate assays, we demonstrated that, in response to DNA damage, DNA-PK interacts with latent p53, specifically phosphorylates the latent population of p53, and is required for DNA-damage-induced apoptosis that is mediated by latent p53. In light of these observations, it was important to assess the significance of Ser15 in the apoptotic mechanism. To this end, MEFs (expressing E1A) derived from a p53 Ser15 to alanine (Ser15→Ala) knock-in transgenic mouse were analyzed for DNA-damage-induced apoptosis. Cells from the parental mouse strain that harbors the wild-type p53 gene had 39% apoptosis following γ -irradiation (Figure 6). The S15A cells had 22% apoptosis. Therefore, in the absence of Ser15 phosphorylation, DNA damage-induced apoptosis was attenuated (by ~44%). This is consistent with a previous report where overexpression of a site-directed mutant p53 (Ser15→Ala) compromised its apoptotic activity (Unger *et al.*, 1999b).

Discussion

We report here that p53-dependent apoptosis in MEFs expressing E1A requires DNA-PK. Previously, it was established that DNA-PK is not required for stabilization of p53, nor is it involved in p53-dependent cell cycle arrest in response to genotoxic stress (Burma *et al.*, 1999; Jimenez *et al.*, 1999; Jhappan *et al.*, 2000). Therefore, DNA-PK selectively regulates the p53-dependent apoptotic response to DNA damage. In addition, it is generally accepted that Atm is involved in p53 stabilization and cell cycle checkpoint functions; however, p53-dependent cell cycle arrest mediated by transcriptional activation is not sufficient for suppression of transformation (Crook *et al.*, 1994). This suggests that the mechanism by which p53 suppresses tumorigenesis is via apoptotic pathways; indeed, p53-dependent apoptosis is a critical regulator of tumorigenesis since inactivation of p53 leads to suppression of apoptosis and rapid tumor growth and progression (Symonds *et al.*, 1994). Therefore, in spite of the role of Atm role in p53-dependent cell cycle checkpoints, mediators of p53-dependent apoptosis, such as DNA-PK, will likely have a greater role in the tumor suppressor activity of p53.

The discovery that DNA-PK plays a part in the p53-dependent apoptotic pathway provides the basis for further analysis of the molecular processes that occur in apoptotic cells. Here we report that DNA-PK_{CS} forms a complex with the latent population of p53 in apoptotic cells, but not in arresting cells. This observation correlates with the fact that inducible p53 is not required for the apoptotic program, and that the 'latent' p53 population is sufficient for apoptosis. Furthermore, DNA-PK is required for phosphorylation of 'latent' p53 at Ser15 in response to γ -irradiation, which correlates with an attenuated apoptotic

Fig. 5. Latent p53 is sufficient for executing DNA damage-induced apoptosis. (A) MEFs expressing E1A were mock-treated (CTRL), treated with cycloheximide (CHX), γ -irradiated (IR) or treated with both cycloheximide and IR (CHX+IR). Five hours following treatment, the cells were stained with calcein AM and Cy3-conjugated annexin V, and mounted in DAPI as a counterstain. Dual-positive stained cells (i.e. staining with both annexin V-Cy3 and calcein) were scored as apoptotic. (B) Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells (as determined by DAPI staining) in each random field (>100 cells) and represent the average of three independent experiments $\pm$ SEM.

response in MEFs expressing the missense mutant form of p53 where Ser15 is mutated to alanine. The fact that these MEFs only have an attenuated apoptotic response compared with an abolished apoptotic response in DNA-PK $_{CS}^{-/-}$ MEFs indicates that DNA-PK does more than just phosphorylate p53 at Ser15. It is reasonable to suggest that there are additional modification or possible conformational changes associated with DNA-PK-p53 complex formation that may enhance the execution of the apoptotic program.

Precisely how latent p53, upon activation by IR-induced DNA damage, mediates the apoptotic response is unclear at present. Tumor-derived p53 is typically mutated in the DNA-binding domain and thus fails to bind to DNA. Given that inactivation of p53 correlates with attenuation of apoptosis and leads to rapid tumor growth and progression (Symonds *et al.*, 1994), it is likely that DNA binding or an alternative activity associated with the DNA-binding domain of p53 may be required for apoptosis. We previously showed that DNA-PK acts upstream of p53

Fig. 6. Ser15→Ala mutant p53 attenuates the DNA damage apoptotic response. MEFs expressing E1A were grown on glass coverslips. Cells were either untreated, or were γ -irradiated (IR). Twenty-four hours following irradiation, the cells were fixed, stained with DAPI and analyzed for morphological characteristics associated with apoptosis. Apoptotic cells were scored for changes in nuclear morphology. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) and represent the average of three independent experiments $\pm$ SEM.

leading to activation of *in vitro* p53 DNA binding (Woo *et al.*, 1998). Naturally, it was presumed that activation of p53 DNA binding *in vitro* would correspond with binding to specific promoters and, therefore, transactivation of target genes *in vivo*. However, since DNA-PK is not required for p53-dependent transactivation of the p21 gene or arrest at G₁ phase of the cell cycle (Burma *et al.*, 1999; Jimenez *et al.*, 1999; Jhappan *et al.*, 2000), *in vitro* p53–DNA-binding activity may instead represent adoption of a conformation that facilitates protein–protein interactions with the DNA-binding domain of p53. In addition to consensus DNA targets, the DNA-binding domain of wild-type but not mutant p53 interacts with 53BP1 and 53BP2 (Iwabuchi *et al.*, 1994). Also, each of the six hotspot p53 mutations that disrupt DNA binding also impair 53BP2 binding (Gorina *et al.*, 1996). Thus, there may be cellular proteins rather than DNA with which p53 must associate for apoptosis and tumor suppressor function. Therefore, the prior demonstration that DNA-PK can activate *in vitro* p53 DNA binding, coupled with the current observation that DNA-PK is required for p53-dependent apoptosis, may represent DNA-PK altering p53 leading to its interaction with another factor via its DNA-binding domain; this may ultimately lead to activation of the apoptotic pathway.

In line with this contention, we previously concluded that activation of p53 DNA binding requires both DNA-PK and an unidentified factor present in DNA damaged cells. We believe this other factor is likely to be Chk2, which was found to be required for p53-dependent apoptosis in MEFs expressing E1A (M.Jack, R.Woo and P.Lee, manuscript submitted). Recently, Chk2 was shown to form a complex with wild-type but not mutant p53 (Falck *et al.*, 2001). Furthermore, activated Chk2 was shown to form foci at DNA strand breaks immediately following exposure to γ -irradiation (Ward *et al.*, 2001). Therefore, it is conceivable that DNA damage-induced apoptosis involves a complex comprising p53, DNA-PK and Chk2, and a series of interactions and phosphorylation events occurring, quite possibly at strand breaks, that ultimately triggers the apoptotic pathway.

Materials and methods

Cell culture and preparation of cell extracts

Early passage MEFs with either wild-type, p53^{+/−} (Donehower *et al.*, 1992) or DNA-PKcs^{−/−} genotype (Kurimasa *et al.*, 1999) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco). To induce DNA damage, cells were irradiated with 5 Gy ionizing radiation using a ¹³⁷Cs irradiator at a rate of ~2.5 Gy/min for 2 min. In the indicated experiments, cycloheximide was used at 30 μ g/ml. For cell extracts, cells were washed with ice-cold phosphate-buffered saline (PBS; 130 mM NaCl, 2 mM KCl, 8.1 mM NaH₂PO₄, 1.5 mM KH₂PO₄), then scraped off the culture plates using a rubber policeman in 1 ml ice-cold PBS. Harvested cells were centrifuged by a quick spin in a microcentrifuge (Eppendorf 5415C). The cell pellet was then resuspended and lysed in approximately two packed cell volumes of ice-cold hypotonic buffer [10 mM HEPES pH 7.6, 10 mM KCl, 5 mM Mg-acetate, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 15 min on ice, followed by repeated passage through a 26-gauge needle to lyse the cell membranes. After centrifugation at 14 000 g for 1 min at 4°C, the supernatant (cytoplasmic lysate) was saved on ice and the pellet was extracted in approximately one packed cell volume of hypertonic buffer (10 mM HEPES pH 7.6, 400 mM KCl, 5 mM Mg-acetate, 1 mM DTT, 10% glycerol, 1 mM PMSF) for 30 min on ice with continuous agitation to prevent the nuclei from settling. The nuclei were pelleted by centrifugation at 14 000 g for 30 min at 4°C, and the supernatant (nuclear extract) was added to the cytoplasmic lysate.

IP and western blotting

Aliquots of cell extracts were pre-cleared with inactivated *Staphylococcus aureus* (IgSorb, The Enzyme Centre). For DNA-PK-related experiments, the hypotonic and hypertonic buffers used to make cell extracts were supplemented with 10 mM MgCl₂, 0.2 mM EGTA and 0.1 mM EDTA. For the pre-clearing step, 30 μ l of a 10% *S.aureus* suspension was pelleted by microcentrifugation. The supernatant was aspirated, the cell extract was added to the pellet, and then incubated for 1 h on ice with the tube being flicked every 5 min to prevent the *S.aureus* from settling. Samples were spun in a microcentrifuge, and the supernatant was transferred to a new tube. If DNase or ethidium bromide was used, the amount of DNA present in the cell extract was estimated spectrophotometrically, and the appropriate amount of DNase (~1 μ g/ml) or ethidium bromide (~10 μ g/ml) was used. Next, the appropriate antibody was added, and incubated on ice for 1 h to overnight. *Staphylococcus aureus* (30 μ l of a 10% suspension) was then added, and the mixtures were incubated for another 45 min. Samples were washed four times with IP buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.05% Tween 20).

For SDS–PAGE, protein samples were boiled for 5–10 min in protein sample buffer (50 mM Tris pH 6.8, 1% SDS, 10% glycerol, 0.01% Bromophenol Blue). β -mercaptoethanol was omitted from the protein sample buffer to avoid interference attributed to cross-reactivity between the secondary antibody used in the immunoblot, and the ~50 kDa IgG heavy chain used in the IP. Electrophoresis was carried out at room temperature, with an applied current of 35 mA for ~3 h.

For western blotting, the proteins were transferred to nitrocellulose for 2 h at 80 V, 4°C. The blot was then rinsed in PBS plus 0.2% Tween 20 (PBS-T), and placed in blocking buffer (5% non-fat milk powder in PBS-T) overnight. Alternatively, Tris-buffered saline (TBS; 20 mM Tris pH 7.6, 137 mM NaCl) plus Tween 20 (TBS-T) would be used in place of PBS-T for the anti-phosphoserine-15 antibody. Next, the blot was incubated in primary antibody, typically at a dilution of 1:2000 in blocking buffer for 1 h to overnight for anti-phosphoserine-15 antibody. Following incubation with the primary antibody, the blot was rinsed twice with blocking buffer, followed by one 15 min and three 5 min washes in blocking buffer on a rocking platform. Anti-mouse (Gibco-BRL) or anti-rabbit (Jackson Laboratories) IgG-HRP secondary antibodies were used at 1:5000 dilution in blocking buffer. The anti-goat IgG-HRP (Santa Cruz) secondary antibody was used at 1:8000 dilution in blocking buffer. Incubations in secondary antibodies were at room temperature for 45 min. This was followed by the same washing regimen as before. The blot was then subjected to luminol-based chemiluminescence (ECL; Amersham), followed by exposure to Kodak X-OMAT film.

Retroviral-mediated gene transfer of the adenovirus E1A 12S oncogene

The $\psi 2$ packaging cell line transfected with the retroviral vector DOL containing the adenovirus E1A 12S cDNA was obtained from ATCC and has been described previously (Cone *et al.*, 1988). These cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. The virus-containing medium was filtered (0.45 μ m filter; Millipore), mixed 1:1 with fresh medium and supplemented with 4 μ g/ml polybrene (Sigma). Target fibroblasts were plated at 50% confluency. For infection, the culture medium was removed and replaced with the virus-containing medium, and incubated at 37°C. The virus-containing medium was replaced with fresh virus every 4 h for 24 h. E1A expression was verified by western blot, and infection efficiency was monitored by anti-E1A immunofluorescent staining (data not shown). At least 90% of the target fibroblasts expressed E1A.

Analysis of apoptotic morphology

Cells grown on cover slips in 60 mm dishes were γ -irradiated to induce apoptosis. The cells were fixed in 2% paraformaldehyde for 15 min. Cover slips were washed with distilled water, then mounted onto slides in 50% glycerol containing 3 μ g/ml of DAPI. Nuclei were visualized using an epifluorescence microscope (Leica). Digital images were collected using a charge-coupled device camera containing a 14-bit detector (Princeton Instruments) then colored with Adobe Photoshop 5.0. Apoptotic cells were scored for changes in nuclear morphology. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells in each random field (>100 cells) and represent the average of three independent experiments $\pm$ SEM.

Annexin V apoptotic assay

Cells grown on cover slips in 60 mm dishes were untreated, treated with cycloheximide (30 μ g/ml), γ -irradiated (5 Gy IR), or pre-treated with cycloheximide for 15 min followed by IR. After 5 h, the cell samples were analyzed with the Annexin V-CY3 Apoptosis Detection Kit (Sigma). Briefly, medium was removed from the cells and replenished with fresh medium containing calcein AM (500 nM). The viability stain in the kit, 6-CFDA, was replaced with calcein AM (Molecular Probes) because the fluorogenic end-product calcein was better retained by the cells. Cells were stained for 10 min, washed three times with PBS then fixed in 2% paraformaldehyde for 15 min. Following fixation, the cells were washed four times with annexin V binding buffer (10 mM HEPES pH 7.6, 140 mM NaCl, 2.5 mM CaCl₂). The cells were then incubated in 1 μ g/ml annexin V-Cy3 for 10 min followed by four washes in binding buffer. The cover slips were then mounted onto slides in 50% glycerol containing 3 μ g/ml of DAPI followed by microscopy as above. Dual-positive stained cells (i.e. staining with both annexin V-Cy3 and calcein) were scored as apoptotic. Apoptotic values were calculated as the percentage of apoptotic cells relative to the total number of cells (as determined by DAPI staining) in each random field (>100 cells) and represent the average of three independent experiments $\pm$ SEM.

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