Chk2 is dispensable for p53-mediated G_1 arrest but is required for a latent p53-mediated apoptotic response

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In response to genotoxic stress, mammalian cells can activate cell cycle checkpoint pathways to arrest the cell for repair of DNA damage or induce apoptosis to eliminate damaged cells. The checkpoint kinase, Chk2, has been implicated in both of these responses and is believed to function in an ataxia telangiectasia (Atm)-dependent manner. We show here that Chk2−/− mouse embryo fibroblasts (MEFs), unlike Atm−/− or p53−/− MEFs, behaved like normal MEFs in manifesting p21 induction and G_1 arrest upon exposure to γ-irradiation. Therefore, Chk2 is not involved in p53-mediated G_1 arrest. To examine the role of Chk2 in p53-dependent apoptotic response, we used adenovirus E1A-expressing MEFs. We show that Chk2−/− cells, like p53−/− cells, did not undergo DNA damage-induced apoptosis, whereas Atm−/− cells behaved like normal cells in invoking an apoptotic response. Furthermore, this apoptosis could occur in the absence of protein synthesis, suggesting that it is preexisting, or "latent," p53 that mediates this response. We conclude that Chk2 is not involved in Atm- and p53-dependent G_1 arrest, but is involved in the activation of latent p53, independently of Atm, in triggering DNA damage-induced apoptosis.

DNA damage | cell cycle control

In response to DNA damage induced by ionizing radiation (IR), eukaryotic cells can activate cell cycle checkpoints or apoptosis. The p53 tumor suppressor mediates these cell responses (1–4), but the mechanism of its activation remains to be fully elucidated. Upstream candidates include the gene mutated in ataxia telangiectasia (ATM), the DNA-dependent protein kinase (DNA-PK), and more recently the checkpoint kinase, Chk2.

Chk2 mutations have been identified in cancer predisposition syndrome Li Fraumeni patients with normal p53 alleles, making it a potential tumor suppressor protein (5–7). Recently, a potential role for Chk2 in the G_1 cell cycle checkpoint has been suggested based on overexpression studies in immortalized or transformed cells (8, 9). It was reported that Chk2 may act downstream of Atm to stabilize and/or activate this p53-dependent response. Although informative, these studies have limitations: (*i*) Overexpressed Chk2 may induce pathways not normally triggered by endogenous Chk2; and (*ii*) immortal and transformed cell lines may harbor mutations that affect Chk2 or Chk2-dependent pathways. How endogenous Chk2 functions in this capacity remains to be clearly shown, but is likely by means of a p53-dependent pathway because Chk2 phosphorylates p53 on Ser-20 (8, 10). Recent studies suggest that Chk2 is also involved in G_2 arrest and apoptosis because Chk2−/− embryonic stem cells fail to maintain IR-induced G_2 arrest and Chk2−/− thymocytes have an attenuated apoptotic response to double-strand break damage (11).

In this study, we used mouse embryo fibroblasts (MEFs) (wild type, p53−/−, Atm−/−, and Chk2−/−) to further dissect the role of Chk2 and its implicated activator, Atm, after DNA damage, in both p53-dependent G_1 cell cycle checkpoint and apoptotic responses. The use of Chk2−/− MEFs should reveal whether in fact endogenous Chk2 plays a role in G_1 cell cycle arrest after IR. For apoptosis studies, the adenovirus E1A 12S gene was introduced into MEFs by retroviral-mediated gene transfer. E1A confers a hyperproliferative state, and the G_1 arrest pathway normally induced after DNA damage is no longer active. Instead, these growth-deregulated MEFs are sensitized to p53-dependent apoptosis (12, 13). It has been established that activating oncogenes including *E1A*, *Myc*, and *E2F* can sensitize MEFs to DNA damage-induced p53-dependent apoptosis (12–16).

Our results show that Chk2 is not involved in DNA damage-induced G_1 arrest because Chk2−/− MEFs have a completely normal arrest whereas Atm−/− cells exhibit an attenuated arrest as previously reported (2). Further, with the MEF E1A model we show that whereas normal MEFs expressing E1A triggers an apoptotic response upon DNA damage, this is not seen in Chk2−/− MEFs expressing E1A, suggesting the absolute requirement for Chk2 in DNA damage-induced apoptosis. In contrast, Atm−/− cells retained the apoptotic response despite a lack of stabilization of p53 protein, indicating that preexisting or "latent" p53 is sufficient to execute the apoptotic response. Use of cyclohexamide (CHX) to block synthesis of new protein confirms that induced and stabilized p53 is not required for induction of apoptosis after IR in MEFs. Thus Chk2 plays an activating role for preexisting p53 in the apoptotic response after DNA damage independent of Atm and is dispensable for the G_1 cell checkpoint.

Materials and Methods

Cell Culture and Preparation of Cell Extracts. Early-passage MEFs with either wild-type, p53−/− (17), Chk2−/− (11), or Atm−/− (18) genotype were maintained in DMEM (GIBCO) supplemented with 10% FBS (GIBCO). To induce DNA damage, cells were irradiated with 5 Gy IR by using a ^{137}Cs irradiator at a rate of ≈2.5 Gy/min for 2 min. For cell extracts, cells were washed with ice-cold PBS, then scraped from the plates into 1 ml ice-cold PBS. Cells were centrifuged and the cell pellet was then resuspended and lysed in approximately two packed cell volumes of hypotonic buffer for 15 min on ice, followed by repeated passage through a 26-gauge needle. After centrifugation the supernatant (cytoplasmic extract) was saved on ice and the pellet was extracted in approximately one packed cell volume of hypertonic buffer for 30 min. The nuclei were pelleted and the supernatant (nuclear extract) was added to the cytoplasmic lysate.

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Abbreviations: IR, ionizing radiation; ATM, ataxia telangiectasia; DNA-PK, DNA-dependent protein kinase; Chk, checkpoint kinase; MEF, mouse embryo fibroblast; PI, propidium iodide; DAPI, 4′,6′-diamidino-2-phenylindole; CHX, cycloheximide.

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RNA Preparation. Total RNA was prepared from MEFs as per the manufacturer's instruction (Qiagen, Chatsworth, CA). RNA was obtained from both untreated MEFs and cells treated with 5 Gy IR (18 h post-IR). Quantitative reverse transcriptase (RT)–PCR was carried out by using 2 μg of RNA for the RT reaction (as per the manufacturer's instruction, Qiagen). p21 primers toward mouse p21 were then used in the PCR to measure p21 RNA levels (5′-CGGTCCCGTGGACAGTGAGCAG-3′ and 5′-GTCAGGCTGGTCTGCCTCCG-3′). Glyceraldehyde-3-phosphate dehydrogenase primers were used on the same RT reaction as a quantitative control for PCR.

Cell Cycle Analysis. For cell cycle analysis, MEFs were examined by BrdUrd labeling in conjunction with FACs analysis. Briefly, MEFs were plated and either mock-treated or irradiated with 5 Gy IR. At the various times indicated, both untreated and irradiated MEFs were pulsed with 10 μM BrdUrd for 3 h. Subsequently, the cells were stained for BrdUrd incorporation with a FITC-conjugated anti-BrdUrd antibody and counterstained with propidium iodide (PI) for analysis by flow cytometry as described (1). The percentage of cells in each phase of the cell cycle and the uptake of BrdUrd was evaluated on a FACscan flow cytometer (Becton–Dickinson).

Western Blotting. For SDS/PAGE, protein samples were boiled for 5–10 min in protein sample buffer (50 mM Tris·HCl, pH 6.8/1% SDS/10% glycerol/0.01% bromophenol blue). Electrophoresis was carried out at room temperature, with an applied current of 35 mA for $\approx$3 h. 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 with 5% nonfat milk powder in PBS-T overnight. Next, the blot was incubated in primary antibody, (FL393; Santa Cruz Biotechnology) at a dilution of 1:2,000 in blocking buffer for 1 h. After incubation with the primary antibody, the blot was thoroughly washed in blocking buffer. Anti-rabbit (The Jackson Laboratory) IgG-horseradish peroxidase secondary antibody was used at 1:5,000 dilution in blocking buffer and incubated at room temperature for 30–45 min followed by washed in blocking buffer. The blot was then subjected to chemiluminescence (ECL; Amersham Pharmacia) and then exposed to Kodak X-Omat film. For protein loading control, a mAb to actin was used 1:10,000 (Sigma) and processing was carried out as described.

Retroviral-Mediated Gene Transfer of the Adenovirus E1A 12S Oncogene. The ψ2 packaging cell line transfected with the retroviral vector DOL containing the adenovirus E1A 12S cDNA was obtained from the American Type Culture Collection and has been described (19). These cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. The virus-containing medium was filtered (0.45-μm filter; Millipore), mixed 1:1 with fresh medium, and supplemented with 4 μg/ml polybrene (Sigma). Target fibroblasts were plated at 50% confluency. For infection, the culture medium was removed, 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). A minimum of 90% of the target fibroblasts expressed E1A.

Analysis of Apoptotic Morphology. Cells grown on coverslips in 60-mm dishes were γ-irradiated to induce apoptosis (5 Gy). 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 μg/ml of 4′,6′-diamidino-2-phenylindole (DAPI). Nuclei were visualized with an epifluorescence microscope (Leica). Digital images were collected by using a charge-coupled device camera containing a 14-bit detector (Princeton Instruments, Trenton, NJ) then colored with Adobe PHOTOSHOP 5.0. Apoptotic cells were scored for changes in nuclear morphology (20). 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 ± SEM.

Annexin V Apoptotic Assay. Cells grown on coverslips in 60-mm dishes were untreated, treated with CHX (30 μg/ml), γ-irradiated (5 Gy IR), or pretreated with CHX for 15 min followed by IR. Seven hours later, 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 fluorigenic 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. After fixation, the cells were washed four times with annexin V binding buffer (10 mM Hepes, pH 7.6/140 mM NaCl/2.5 mM $CaCl_2$). The cells were then incubated in 1 μg/ml annexin V-Cy3 for 10 min followed by four washes in binding buffer. Coverslips were then mounted onto slides in 50% glycerol containing 3 μg/ml 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 ± SEM.

Results

Chk2 Is Not Required for p53-Dependent G_1 Cell Cycle Arrest. It has been established that p53 and Atm are required for a normal cellular G_1 arrest after irradiation (3). Because Chk2 has been implicated in the G_1 cell cycle checkpoint and predicted to act downstream of Atm in this pathway, the role of endogenous Chk2 in this capacity was examined *in vivo*. To examine the G_1 arrest checkpoint, MEFs (wild type, p53−/−, Atm−/−, and Chk2−/−) were mock-treated or exposed to IR followed by BrdUrd and PI labeling at 12, 24, and 30 h thereafter. Samples were then fixed, processed with anti-BrdUrd FITC-conjugated antibody and PI stain, and analyzed by flow cytometry. Only early-passage (four or earlier) MEFs were used for these analyses to ensure normal karyotype and morphology. Atm−/− MEFs were examined only at passage 1 because these MEFs enter a senescent-like state at passages 2 or 3 (18). As shown in Fig. 1 *a* and *b* (only the 24-h samples are presented in Fig. 1*a* for clarity of presentation), wild-type MEFs exhibited a normal G_1 arrest and an approximately 80% decrease in BrdUrd incorporation (because of failure to enter the S phase) in response to exposure to IR. Control cells that were mock-treated all underwent normal cell cycling and incorporated BrdUrd, reflecting the number of cells in S phase in these random populations (data not shown). In contrast, p53−/− MEFs lost the ability to arrest in G_1 after IR, and Atm−/− MEFs had an attenuated arrest as reported (3, 21, 22). However, the attenuated response in the Atm−/− MEFs was not as pronounced as expected (as compared with p53−/− MEFs). Most interestingly, the Chk2−/− cells retained the ability to arrest the cell cycle at G_1 after IR, with corresponding decrease in DNA synthesis (as indicated by the drastically reduced BrdUrd incorporation) and in a time-dependent manner comparable to that observed with wild-type cells. These results demonstrate that loss of Chk2 does not abrogate the G_1 checkpoint after IR and that Chk2 does not act downstream of Atm to mediate this response.

To confirm that Chk2−/− MEFs arrest at G_1 in a p53-

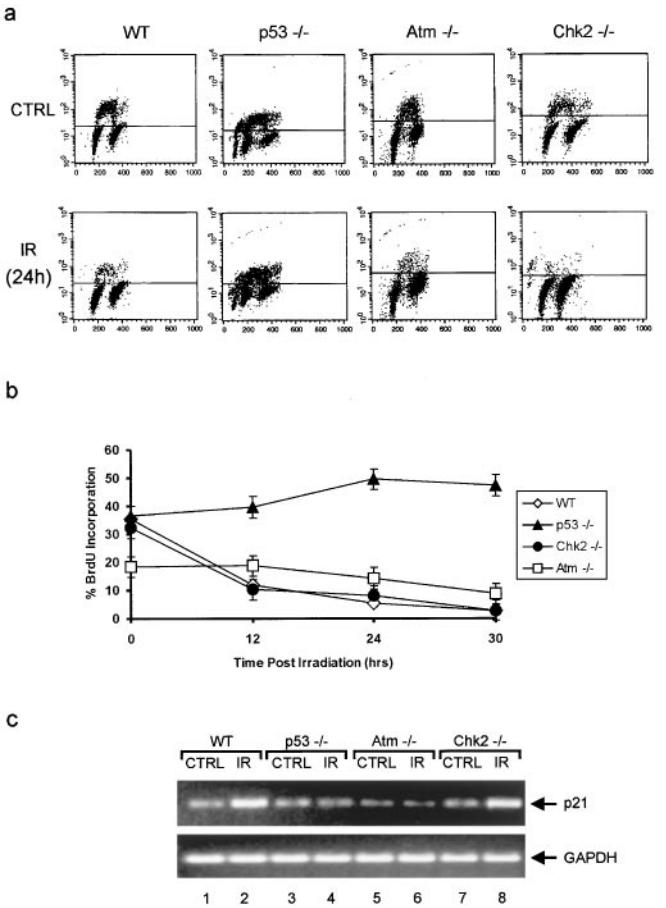

Fig. 1. Chk2 is dispensable for p53-dependent G_1 cell cycle arrest. MEFs at early passage were plated and either mock-treated or irradiated and BrdUrd-labeled at various times postirradiation. (*a*) Representative flow-cytometry scatter plots of wild-type (WT), p53−/−, Chk2−/−, and ATM−/− MEFs, either control (CTRL) or 24 h postirradiation, were plotted after labeling with BrdUrd as increasing fluorescence of PI (*x* axis) versus increasing FITC fluorescence obtained with an anti-BrdUrd FITC-conjugated antibody to detect BrdUrd incorporation (*y* axis). (*b*) Quantitative assessment of the percentage of MEFs in S phase, as indicated by BrdUrd incorporation, before and after the various time points postirradiation for each genotype are graphed as an average of three independent experiments ± SEM. (*c*) Quantitative reverse transcriptase–PCR was used to examine endogenous expression of p21 in wild-type, Chk2−/−, p53−/−, and Atm−/− MEFs before and after irradiation. To ensure equal RNA in the reactions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were also assessed.

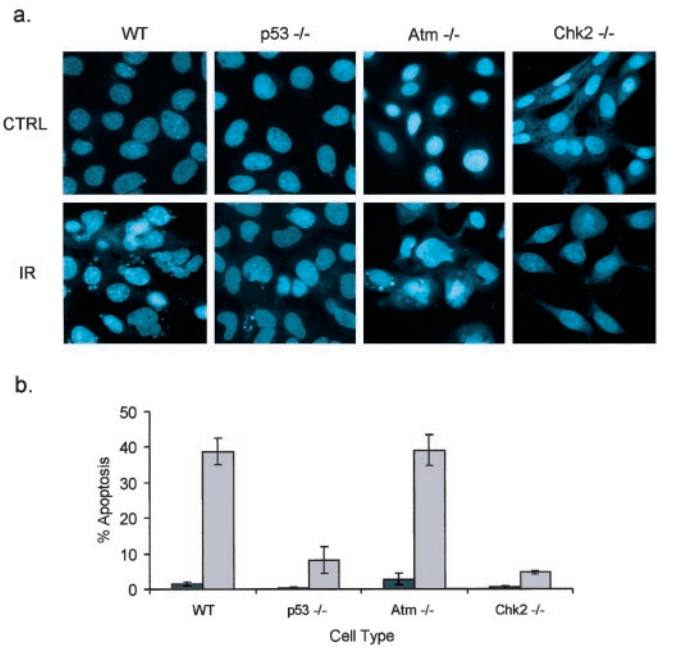

Fig. 2. Factors involved in oncogene-sensitized, p53-dependent apoptosis. MEFs [wild type (WT), p53−/−, Chk2−/−, and Atm−/−] expressing E1A were grown on glass coverslips. Cells were either untreated (CTRL) or γ-irradiated (IR). (*a*) Twenty-four hours after irradiation, the cells were fixed, stained with DAPI, and analyzed for morphological characteristics associated with apoptosis. (Magnification: ×400.) (*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 ± SEM.

dependent manner, p21 mRNA expression before and after 5 Gy IR was evaluated by quantitative PCR (Fig. 1*c*). Basal levels of p21 mRNA were relatively similar in all genotypes tested and, after IR treatment, p21 was induced in wild-type MEFs as expected. Induction of p21 was not observed in p53−/− MEFs, confirming the p53 dependence of this response. [The difference in p21 expression between these cells was also evident at the protein level as p53−/− MEFs did not have any detectable increase in p21 protein levels (data not shown).] Lack of p21 induction was also evident in Atm−/− MEFs, which may explain the attenuated cell growth arrest seen with these cells. In contrast, activation of p21 expression was clearly demonstrable after DNA damage in Chk2−/− cells, confirming that Chk2 is not involved in p53/p21-dependent G_1 arrest.

Chk2 Is Required for p53-Mediated Apoptosis *in Vivo*. Our demonstration of the lack of Chk2 involvement in the G_1 checkpoint led us to wonder whether Chk2 could play a role in p53-dependent apoptosis. To this end, MEFs were first sensitized to this response by introducing the adenovirus E1A 12S oncoprotein by retroviral gene transfer. Cells plated on glass coverslips were mock-treated or irradiated with 5 Gy. At 24 h post-IR, the control and treated cells were fixed and examined by DAPI staining for morphological changes associated with apoptosis, including nuclear blebbing and heterochromatin aggregation (20) (Fig. 2*a*). Quantitative analysis of the apoptotic response is displayed in Fig. 2*b* and represents the average of three independent experiments with a minimum of 100 cells counted in each experiment. As expected, untreated cells of all genotypes examined did not have any morphological characteristics of apoptosis (Fig. 2), illustrating that E1A alone cannot induce apoptosis. As well, MEFs without E1A did not exhibit apoptotic morphology after irradiation, demonstrating that E1A is required to sensitize these cells to apoptosis (data not shown). Whereas wild-type cells exhibited 39% apoptosis after IR, p53−/− cells failed to undergo apoptosis, reconfirming the p53 dependence of the apoptotic response in this system. Importantly, Chk2−/− cells also failed to induce apoptosis after low-dose irradiation. These results therefore clearly demonstrate that Chk2 is involved in p53-mediated apoptotic response of MEF cells to irradiation.

Atm Is Dispensable for DNA Damage-Induced Apoptotic Response in MEFs Expressing E1A. A number of studies have been conducted regarding the role of Atm in activating Chk2 after irradiation (9, 23, 24). It has been shown that Chk2 undergoes phosphorylation after IR and specifically Thr-68 requires Atm for phosphorylation after DNA damage. To address the potential role of Atm acting upstream of Chk2 in the apoptotic response to IR, wild-type and Atm−/− MEFs expressing E1A were examined

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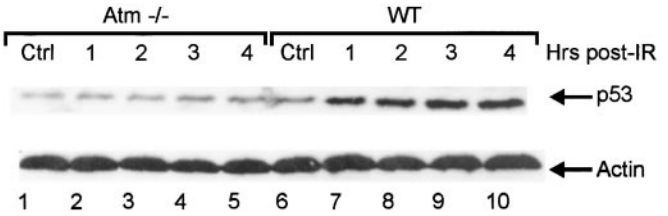

Fig. 3. Comparing wild-type (WT) and Atm−/− MEFs (both expressing E1A) in terms of p53 stabilization upon IR. Wild-type or Atm−/− MEFs expressing E1A were examined for their ability to stabilize p53 after irradiation. Cell extracts were prepared from unirradiated or IR (5 Gy)-treated cells collected at various time intervals. Immunoblots (75 μg protein/lane) of cell extract were probed with a polyclonal antibody (FL393) against p53. To confirm equal protein loading in each lane, the amount of actin was also assessed by using an antiactin antibody. Ctrl, Control.

as described above. As mentioned earlier, Atm−/− MEFs enter an early senescence at passages 2 or 3. As a result, all experiments are done ideally with passage 1 cells. With E1A, this early senescent phenotype is overcome, and the cells proliferate normally. These cells were again examined for their apoptotic response to IR by DAPI staining and morphological assessment. As seen in Fig. 2, Atm−/− cells retain the ability to apoptose. This finding suggests that Atm is dispensable for the apoptotic program, and combined with the Chk2−/− data, further infers that Atm is not acting upstream of Chk2 to trigger p53-dependent apoptosis. Atm is widely accepted as necessary for p53 stabilization. To verify whether p53 protein was stabilized in the Atm−/− E1A-expressing cells, p53 protein levels were examined at various times postirradiation. As shown in Fig. 3, p53 is not stabilized in these Atm−/− cells (compare lanes 1–5 and 6–10). Because we have demonstrated that these cells retain the apoptotic response to IR, stabilization of p53 is not required for this DNA damage-induced response. These results argue for a role of nonstabilized p53 as sufficient to execute the programmed cell death pathway. Further, they suggest that Chk2 is not acting to stabilize p53 in this response but rather is functioning in an activating role.

Chk2 Acts with Latent p53 to Activate the Latent p53 Response *in Vivo*. If stabilization of p53 is unnecessary for IR-induced apoptosis, then the preexisting, unstabilized p53 population may be sufficient to mediate this response *in vivo*. Therefore, experiments were undertaken to determine whether the apoptotic pathway involving Chk2 and p53 is mediated by the preexisting or uninduced p53 population. To this end, wild-type MEFs expressing E1A were plated on glass coverslips and irradiated and treated with CHX alone or with CHX followed by irradiation or mock treatment. Treatment with CHX inhibits translation of any new protein, including p53. Thus, the p53 response being measured depends on the existing p53 or latent p53 present at the time of irradiation. To assay for apoptosis, a biochemical approach using annexin V to detect an early apoptotic marker was used in conjunction with the viability marker calcein AM. Calcein AM is nonfluorescent unless hydrolyzed in viable cells to become a fluorescent florescien derivative. Annexin V interacts with phoshphatidyl serine, which is translocated from the cytoplasmic surface of the cell to the cell surface early in the apoptotic response (25). This approach was necessary because CHX may induce necrosis in cells upon prolonged treatment (e.g., after 24 h) so that late morphological markers would be difficult to interpret. Annexin V staining in conjunction with viability stain was carried out at 7 h postirradiation. As Fig. 4 clearly shows, the latent p53 population is sufficient to mediate the apoptotic response after irradiation. Apoptotic cells are those that are both viable (green) and annexin V-positive (red).

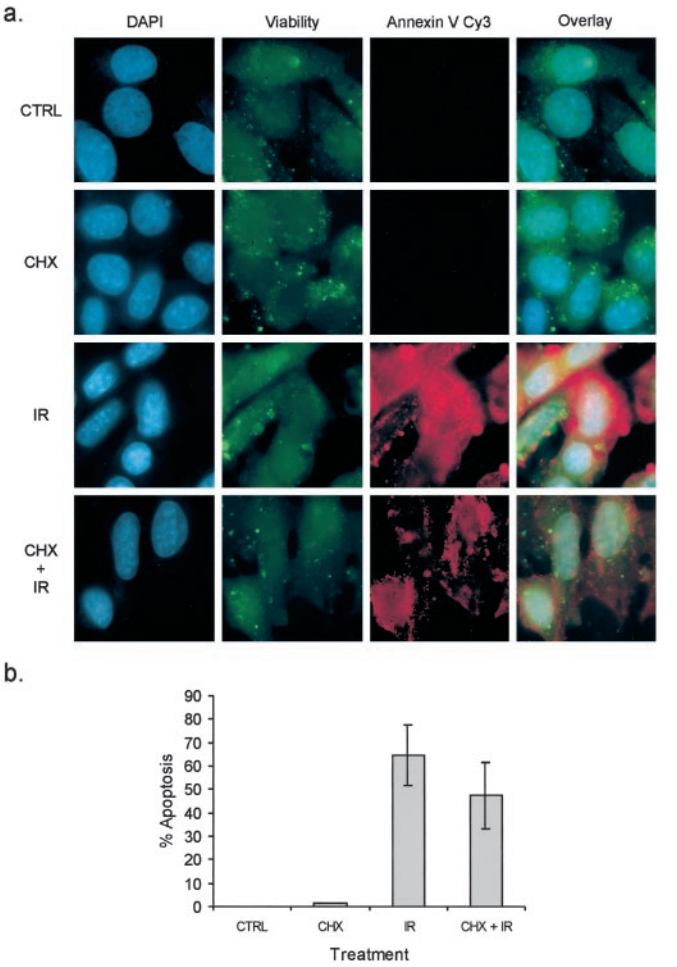

Fig. 4. Preexisting, nonstabilized p53 is sufficient for execution of DNA damage-induced apoptosis. (*a*) Wild-type MEFs expressing E1A were mock-treated (CTRL), treated with CHX, γ-irradiated (IR), or treated with both CHX and IR. Seven hours after treatment, the cells were stained with calcein AM and 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. (Magnification: ×800.) (*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 ± SEM.

Control cells and CHX-treated cells did not induce annexin V staining and therefore did not activate the apoptotic program. In both the irradiated samples and in the samples treated with CHX before irradiation, cells exhibited an apoptotic response of 65% and 48%, respectively. This finding clearly demonstrates that despite the lack of synthesis of new protein the preexisting p53 is sufficient to mediate the DNA damage-induced apoptotic response. This experiment was also carried out on p53−/− cells and neither the CHX, IR, or CHX with IR treatments induced apoptosis (data not shown), demonstrating that this response depends on both p53 and DNA damage.

Discussion

MEFs provide a valuable tool with which to study both the G_1 arrest and apoptotic activities of p53. G_1 arrest is clearly p53-dependent because p53−/− MEFs fail to induce G_1 arrest after DNA damage (3). In contrast, MEFs expressing the adenovirus E1A oncoprotein are hyperproliferative and are reprogrammed to induce p53-dependent apoptosis after DNA damage or stress (12, 13). Using MEFs as an *in vivo* model system to study both

p53 responses, we were able to evaluate the potential role of Chk2 and its implicated upstream activator Atm in DNA damage-induced G_1 arrest and apoptosis.

Our results indicate that Chk2 is not required for G_1 arrest after IR and thus have eliminated the potential that Atm may be acting via Chk2 to mediate this cell cycle checkpoint. In this respect Atm may be acting directly at Ser-15 on p53 as a kinase or may be activating an as-yet-unidentified factor to activate p53 G_1 arrest. Previously, Chehab *et al.* (8) had demonstrated that *in vivo* ectopic expression of wild-type Chk2 led to increased p53 stabilization in a human cell line and that this resulted in G_1 arrest after DNA damage. In the present study, however, we used Chk2−/− MEFs and looked at the endogenous Chk2 and p53 response specifically in the absence or presence of the Chk2 and p53 genes. Whereas overexpression of Chk2 protein may lead to cellular arrest, endogenous levels are clearly not required for the normal induction of the G_1 checkpoint.

In addition to cell cycle arrest, p53 can induce apoptosis in response to DNA damage. The Chk2 kinase has also been implicated in this pathway. Hirao *et al.* (11) demonstrate that Chk2−/− thymocytes have an attenuated apoptotic response, resembling that of p53−/− thymocytes. It was suggested that this pathway may require Atm but there has yet to be data reported to illustrate this notion. The role of Atm in apoptosis has been controversial. Atm has been shown to be dispensable for p53-dependent apoptosis in thymus tissue, thymocytes, and the choroid plexus epithelium of a transgenic mouse brain tumor model (21, 26, 27). Alternatively, evidence has also been presented that shows that apoptosis of neurons in the developing central nervous system is Atm-dependent (28) and that there is a partial apoptotic reduction in Atm−/− thymus cells or thymocytes (22, 29).

Using MEFs expressing E1A, we demonstrate that Chk2 is also essential for oncogene-sensitized p53-dependent apoptosis after IR. Furthermore, we show that the preexisting, unstabilized population of p53, and not DNA damage-induced p53, is sufficient for activation of the apoptotic response. We have demonstrated that Atm−/− cells retain the apoptotic response despite the inability to stabilize p53. Interestingly, we have also observed Thr-68 phosphorylation of Chk2 in Atm−/− cells after IR (data not shown), suggesting that perhaps Chk2 is phosphorylated at this residue by another kinase. It will be of interest to determine whether phosphorylation of Thr-68 is required for the apoptotic response. Our results clearly show that Atm is not upstream of Chk2 and that stabilization of p53 is not required for oncogene-sensitized apoptosis in MEFs. This finding illustrates a role for Chk2 in an activating capacity toward p53 in addition to its role as a stabilizer of p53 as suggested in previous studies. Chk2 has been shown to phosphorylate p53 at various sites in the amino terminus (8, 10). In particular, a role for Chk2 in stabilizing p53 by means of Ser-20 phosphorylation has been implicated (10). As stabilization has been shown here to be dispensable for this p53 response it will be necessary to resolve whether p53 Ser-20 has an activating role in this response.

It has recently been reported that Chk2 forms foci at DNA strand breaks immediately after double-strand break DNA damage (30). Interestingly, we have recently demonstrated that DNA-PK forms a complex with p53 immediately after IR and that DNA-PK phosphorylates the latent form of p53 at Ser-15 within minutes of DNA damage (31). Further, DNA-PK−/− cells were also shown to have an abolished apoptotic response as seen here with the Chk2−/− cells. Taken together, these data implicate a potential apoptotic pathway in which Chk2, DNA-PK, and latent p53 interact immediately after DNA damage, where a series of interactions and phosphorylation events at double-strand breaks trigger p53-mediated apoptosis.

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