The Ability of p53 to Activate Downstream Genes p21<sup>WAF1/cip1</sup> and MDM2, and Cell Cycle Arrest following DNA Damage Is Delayed and Attenuated in scid Cells Deficient in the DNA-dependent Protein Kinase*

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scid mouse embryonic fibroblasts are deficient in DNA-dependent protein kinase activity due to a mutation in the C-terminal domain of the catalytic subunit (DNA-PKcs). When exposed to ionizing radiation, the increase in levels of p53 was the same as in normal mouse embryonic fibroblasts. However, the rise in p21<sup>WAF1/cip1</sup> and mdm2 was found to be delayed and attenuated, which correlated in time with delayed onset of G1/S arrest by flow cytometric analysis. The p53-dependent G1 checkpoint was not eliminated: inactivation of p53 by the E6 protein in scid cells resulted in the complete loss of detectable G1/S arrest after DNA damage. Immunofluorescence analysis of normal cells revealed p53 to be localized predominantly within the cytoplasm prior to irradiation and then translocate to the nucleus after irradiation. In contrast, scid cells show abnormal accumulation of p53 in the nucleus independent of irradiation, which was confirmed by immunoblot analysis of nuclear lysates. Taken together, these data suggest that loss of DNA-PK activity appears to attenuate the kinetics of p53 to activate downstream genes, implying that DNA-PK plays a role in post-translational modification of p53, without affecting the increase in levels of p53 in response to DNA damage.

The mechanism by which the signal of DNA damage is transduced to p53 remains to be defined. Initially, it had been presumed that the effect of the signal from DNA damage was to increase the amount of p53 protein in the cell, which could then signal other genes in the damage response pathway (6). More recently, it has been reported that p53-dependent transactivation can occur in the absence of any increase in the level of p53 (7). In addition, Haapajarvi et al. (8) demonstrated that the effect of ultraviolet light exposure on cells synchronized in G1 was to increase transcriptional activity of p53 and subsequently G1 arrest, well before any demonstrated increase in the levels of p53 protein. Furthermore, the accumulation of p53 only occurred when the cells had progressed into S-phase of the cell cycle. Thus, it seems likely that the signal from DNA damage can affect both the levels of p53 and the active state of p53 (post-translational modification). These two responses from DNA damage to p53 could share a common signaling pathway or utilize different pathways, as there has been no genetic basis to separate them to date. Most recently, the use of protein kinase C inhibitors resulted in increased levels of p53 (secondary to an increase in its half-life) and consequent translocation to the nucleus. However, DNA damage was still needed to increase the activity of p53, as measured by a β-galactosidase transactivation reporter assay (9).

The timing of the response to DNA damage does not provide a definitive answer to discriminate between post-translational modification and elevated levels of p53. The levels of p53 are seen to rise by 1–2 h after exposure to IR and reach a peak between 2 and 6 h, depending on the cell type tested. The levels of MDM2 are found to increase within 2–4 h of IR (10) and p21 shows a similar time course (3–11). The fact that downstream genes are transcriptionally activated and the resultant protein detected by 2 h suggests that the rapid response to p53 is mediated by a post-translational modification of p53 or modification of an inhibitory protein such as MDM2, which occurs more rapidly than a rise in the level of p53.

The DNA-activated (or dependent) protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated in vitro by DNA fragments (12, 13). Several observations have suggested that a large polypeptide (initially reported to be 350 kDa, but now thought to be approximately 460 kDa (14)) is the catalytic subunit of DNA-PK, while a cellular DNA end-binding protein, the Ku autoantigen (consisting of 70- and 80–86-kDa proteins), serves as the regulatory subunit (13, 15, 16). DNA-PK has been shown to phosphorylate several nuclear DNA-binding proteins in vitro, including the transactivation domain of p53 (17). Recently, these observations were expanded to show the importance of serines 15 and 37 in mediating the response to DNA damage in vivo, and showing these sites were phosphorylated in vitro by DNA-PK (18). Furthermore, serine 15 of p53 has been shown to be one site which is.

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* The abbreviations used are: IR, ionizing radiation; DNA-PK, DNA-activated protein kinase; ATM, mutated in ataxia telangiectasia; MEF murine embryonic fibroblasts; PBS, phosphate-buffered saline; Gy, gray; CMV, cytomegalovirus.
phosphorylated in response to DNA damage (19). Mutation of the serine 15 phosphorylation site of human p53 to alanine demonstrated impaired transactivation of p53 and, as a consequence, impaired cell-cycle arrest at the G1/S transition (20). However, Shieh et al. (18) made reference to unpublished data showing constitutive and induced phosphorylation of p53 at serine 15 in murine scid cells, implying that other pathways distinct from DNA-PK can phosphorylate p53 at serine 15 in response to DNA damage. The ATM kinase is also a candidate kinase for phosphorylation of serine 15, but ATM-deficient cells also show induced phosphorylation at this site implying parallel pathways to signal stress, such as DNA damage, via phosphorylation of serine 15 of p53. More recent studies have shown that adding back the ATM gene in these deficient cells results in increased phosphorylation at the serine 15 site, using a specific phosphoserine antibody (21).

The mouse homologue of the human gene, encoding the catalytic subunit of DNA-PK, has been found to be mutated in the scid (severe combined immunodeficiency) gene locus (22, 23). The mutation has been characterized as a short deletion in the C-terminal domain of the protein, which destabilizes the protein and results in a low level of measurable DNA-PK activity (24). The mouse scid is a recessive, autosomal mutation that results in the inability to produce mature, functioning lymphocytes because of failure in V(D)J recombination (25, 26). scid mice and scid cells in culture have been shown to be highly sensitive to IR and other agents that induce DNA double-strand breaks, suggesting that the scid gene product plays a role in DNA repair (27–30). The other two gene products, Ku80 and Ku70, which together with the scid gene product (DNA-PKcs) constitute the DNA-PK holoenzyme, have also been shown to be involved in the same response pathways, since cells containing mutant or absent Ku 80 or Ku 70 are sensitive to IR and deficient V(D)J recombination (31–33).

A number of recent reports have shown that the p53 response and the G1/S cell cycle checkpoint in murine scid cells is normal (34–38), casting serious doubt about any connection between p53 and DNA-PK. However, none of these publications looked in detail at the kinetics of cell cycle arrest or the timing of transactivation of p53-dependent downstream genes by observing multiple time points following exposure to ionizing radiation or other DNA-damaging agent. Furthermore, DNA damage-induced phosphorylation of serine 15 of p53 results in alleviation of the inhibition from MDM2 (18). More recently, it has been shown that DNA-PKcs is necessary for activation of DNA binding by p53 (in response to IR) in a gel shift assay (39). Failure to phosphorylate p53 could produce the phenotype of scid cells: an attenuated and perhaps delayed response proportion to the elevation of levels of p53, without the rapid response brought about by loss of inhibition by MDM2. The effect of DNA-PK could be directly on p53 or indirectly on an inhibitory protein such as MDM2. It has recently been suggested that DNA-PK might phosphorylate MDM2, which then prevents inhibition of p53 (40). These observations are not mutually exclusive, and therefore the effect of loss of DNA-PK activity could be a combined effect of lack of phosphorylation of p53 (resulting in impaired transactivation) and lack of phosphorylation of MDM2 which then continues to attenuate p53 (rather than phosphorylation which results in loss of inhibition of p53).

Thus, the exact role of DNA-PK in vivo remains to be defined, as it appears to function both in DNA repair and in sensing DNA damage (41). The gene mutated in patients with ataxia telangiectasia (ATM) produces a protein which is related to DNA-PKcs. Cells containing mutant or absent ATM have previously been described to have an abnormal response of p53 to IR (42). Although the precise nature of the p53 response in ataxia telangiectasia cells has been debated (42, 43) there now appears to be a consensus that the response is delayed and attenuated (44, 45). Therefore, we investigated the DNA damage response pathways in mouse scid cells in order to ascertain whether DNA-PK can regulate the function of p53 in vivo.

**MATERIALS AND METHODS**

**Cell Culture, DNA Transfer, and Growth Selection—**p53 function was analyzed in two murine cell cultures: primary murine embryonic (day 12–20) fibroblasts (MEF) from a normal mouse (FC) and from a Balb/C derived scid mouse (PS). The primary cultures were established directly from the mouse embryos obtained from the Edwin L Steele Laboratory, Massachusetts General Hospital, as described previously (46). These cells were cultured in Dulbecco's modified Eagle's medium at 37 °C in humidified air with 5% CO2. The medium was supplemented with 1 mg/ml glucose, 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 20 mmol of Hepes solution (Sigma). The cells were maintained in exponential growth in monolayer cultures, and used within 10–12 passages from their initial establishment in cell culture. If cells were maintained in cell culture for more than 20 passages, a significant incidence of developing abnormal p53 function was observed (47). Retroviral constructs containing the E6 protein of human papilloma virus type 16/18 (pXI-PoE6; A. J. Levine, Princeton (50)). In summary, 50 μl of retrovirus supernatant was transferred into FC and PS cells, and cells containing a stable integration of the vector were selected by growth in G418 (0.5 mg/ml; Life Technologies, Inc.).

**Cell Irradiation—**Murine embryonic fibroblasts from normal and scid mice were plated in 150-mm dishes and grown to 50–70% confluence at the time of irradiation. The tissue culture medium was renewed 1 day prior to irradiation to remove non-viable unattached cells. Ionizing radiation was delivered using either a 60Co source (Theratron) or a linear accelerator delivering 4MV x-rays (Varian).

**Flow Cytometry and Cell Cycle Analysis—**The cell cycle at the G1/S transition in response to IR was measured using flow cytometry as described previously (48). In brief, exponentially growing cells (1–5 × 106) were irradiated with 8 Gy IR and collected at specific time points (0, 3, 6, 9, 12, and 24 h) following irradiation. The cells were harvested from the culture dishes using trypsin/EDTA, washed twice in phosphate-buffered saline (PBS) before suspension in 0.75 ml of ice-cold 70% ethanol, and incubated on ice for 1 h. The cells were pelleted, washed a further two times in PBS, and then exposed to propidium iodide (100 μg/ml) and RNase A (500 μg/ml). DNA analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) emitting a 488-nm beam (26). The G1/S transition was evaluated by the decrease in the proportion of S-phase cells coupled with an accumulation of cells in G1, with time after irradiation.

**Cell Lysates—**Cells were harvested and lysed at 0, 3, 6, 9, 12, and 24 h after 8 Gy irradiation, in parallel with the flow cytometric analysis. For each time point, cells were washed twice in ice-cold PBS, then 1 ml of ice-cold lysis buffer (1% Triton X-100, 10 μM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM Aprotinin) was added and cells were scraped from the plates. The cell suspension was incubated on ice for 30 min and then centrifuged for 10 min at 13,000 × g at 4 °C. Supernatant was then removed and protein levels quantified using the BCA protein quantification solution. Whole cell lysates were stored at −70 °C.

Preparation of nuclear lysates, at the same time points as the collection of whole cell lysates, used the method described by Price and Calderwood (49). In brief, cells were first washed twice in ice-cold PBS, then 0.5 ml of nuclear lysis buffer (20 mM Hepes, pH 7.8, 20% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100) was added and cells were scraped from the plates. The cell suspension was incubated on ice for 5 min and then spun down at 2,000 × g at 4°C. Supernatant was then removed and pelleted was re-suspended in 400 μl of nuclear extraction buffer (20 mM Hepes, pH 7.8, 20% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 500 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100). After incubation on ice for 15 min, the mixture was spun at 25,000 × g for 15 min. The supernatant (containing nuclear proteins) was then removed and protein quantified. The lysates were stored at −70 °C.

**Western Analysis—**After protein quantification, Western analysis (immunoblot) was carried out using standard procedures with the use of antibodies against p53 (pAb 421-Oncongene Science), p21 (Santa Cruz), and mouse MDM2 (2A10, A. J. Levine, Princeton (50)). In summary, 50
**RESULTS**

**Cell Cycle Arrest following DNA Damage Is Delayed in Scid Fibroblasts**—Normal fibroblasts with wild-type p53 function arrest in the G1/S phase of the cell cycle when exposed to IR (42, 51, 52). This response is attributable to the activation of p53 by radiation-induced DNA damage or similar lesions. If DNA-PK participates directly in DNA damage recognition and transmits this signal to p53, the *scid* (DNA-PK catalytic subunit deficient) fibroblasts may show an abnormality in G1/S arrest.

Fibroblasts isolated from mouse *scid* lines (FS) as well as normal mouse fibroblasts (FC), both of which contain wild-type p53, were exposed to 8 Gy IR. The effect on cell cycle progression was demonstrated by the accumulation of cells at the G1/S checkpoint, which occurred at 6–9 h in FC cells, as shown in Fig. 1A. The kinetics of this normal response has been previously described (1). However, a delayed and attenuated G1 arrest was noted in the FS fibroblasts, which showed little reduction in the S-phase fraction at 9 h, and only by 24 h was there evidence of accumulation of cells in G1 and diminution of cells in S-phase, as shown in Fig. 1B.

To demonstrate that *scid* cells have residual function of p53, pX1PheoE6 was transfected and maintained in both normal and *scid* MEF cells. The transfected cells then expressed little or no detectable p53 (data not shown), as has been shown by multiple previous reports. Fig. 1C shows the flow cytometric profile in response to DNA damage in *scid* cells with E6 protein. At 9 h after 8 Gy IR, there is a lack of any detectable accumulation of cells in G1 and no decrease in the proportion of S-phase cells. The lack of G1 and large accumulation of cells in G2 makes the estimation of S-phase more difficult, but the most conservative model to fit the data supports the lack of decrease in S-phase component. At 24 h, cells remain detectable throughout G2 and S-phase, again suggesting the loss of any G1/S checkpoint in response to DNA damage. The flow cytometric profile of FC-E6 in response to IR was essentially the same as FS-E6 (data not shown). Both FS and FC cells have been transfected with a CMV based expression vector containing a dominant negative mutation of p53, alanine 143, and neither cell line shows a G1/S arrest or p21WAF1/CIP1 response (data not shown) confirming that the responses are p53-dependent.

**The Rise in Level of p53 Is Normal in Scid Fibroblasts**—An increase in p53 protein levels appears to be closely linked to the IR-induced G1 arrest. Since *scid* fibroblasts lack the normal arrest kinetics in DNA synthesis after exposure to IR, the relationship between the *scid* phenotype (deficient in DNA-PK activity) and p53 function was investigated. Immunoblotting of the lysates from FS and FC cell cultures at times following exposure to 8 Gy IR is shown in Fig. 2A. The response of p53 to DNA damage showed only a 3-fold increase at 3–6 h and was not significantly different between FS and FC lines. Although the cell cycle arrest kinetics were delayed in the *scid* MEF, the rise and fall of p53 appeared to be normal. Interestingly in *scid* cells, there is a second rise in p53 levels at 24 h. These findings were reproduced in three independent experiments and was thought to be due to persistent DNA breaks. The conclusion is that DNA-PK does not affect the regulation of p53 levels, which is largely determined by degradation processes.

**p21 and MDM2 Induction Is Delayed and Attenuated in Scid Fibroblasts**—Analysis of the same cell lysates from FS and FC for induction of p21, revealed that the rise of p21WAF1/CIP1 was attenuated and delayed in FS cells. Fig. 2B shows a 3-fold increase in p21WAF1/CIP1 in the *scid* MEF, FS, which was seen maximally at 12–24 h. It is noted that the p21 band in FS cells appears to be slightly shifted below the control band. However, the stringency of the blot suggests a specific, albeit weak, signal. No time points beyond 24 h were evaluated, although cells which continue to grow after irradiation show that...
t* 0 3 6 9 12 24 + - p53

A

 FS

 FC

 control

 pAb421.

B

 t* 0 3 6 9 12 24 + - p21

FS

 FC

 control

 Fig. 2. Immunoblot of whole cell lysates from FS (upper panel) compared with FC (lower panel) cells. Time points are shown from immediately prior to irradiation, then every 3 to 12 h, and finally at 24 h. Fifty micrograms of protein were loaded per lane. A, p53 using pAb421. The positive control was SAOS-2 cells with wild-type p53 transiently transfected, the negative control was SAOS-2 alone, which contains no p53. B, p21WAF1/cip1. The positive control was a rat embryo fibroblast after 8 Gy IR, and the lane marked (−) was for the same cell prior to IR exposure. C, MDM2. The positive control was SAOS-2 cells with transient transfection of pCMV-MDM2. The 90-kDa position of MDM2 was studied in FS cells and found to be attenuated and decreased, which was observed in their original characterization (53), but it is not known what these two bands represent. The ratio of the two bands does not appear to change with DNA damage in either cell type, and thus there is nothing to link the different bands to post-translational modification. In contrast to FS, the normal FC cell showed 5-fold induction of MDM2 by 3 h, which is at least coincident with or even preceding the p53 response. Taken together, these two gene products, p21WAF1/cip1 and MDM2, which are known to be transactivated in a p53-dependent manner provide evidence that the p53 response to DNA damage is not normal in scid cells with deficient DNA-PK activity.

Nuclear Accumulation of p53 Is Abnormal in Scid Cells—Nuclear lysates were examined for the levels of p53, MDM2, and p21 following exposure to IR. Fig. 3A shows the rise and fall of nuclear p53 in FS and FC cells. As seen in whole cell lysates, there was no deficit in the ability of scid cells to sustain a response to IR by an increase in level of p53. Furthermore, the level of p53 in scid cells rises by 3–6 h, falls by 9 h, and then rises again by 24 h, in contrast with FC cells which show little secondary rise at 24 h. However, the most striking difference between the cells was the significantly higher levels of p53 found in the nucleus of scid cells at all time points (shown in Fig. 3B). The regulation of p53 between the cytoplasm and nucleus appears to be abnormal in scid cells, and this was confirmed by immunofluorescence (Fig. 4). Prior to irradiation, p53 was found to be localized predominantly within the cytoplasm in normal FC cells; following IR, p53 was found to translocate to the nucleus (as reported previously (54, 55)). In scid cells, p53 was found at high levels in the nucleus prior to irradiation.

Nuclear MDM2 Shows Abnormal Response to IR in Scid Cells—The levels of MDM2 in nuclear lysates were also found to be abnormal in scid cells compared with normal FC cells. Prior to irradiation, detectable levels of MDM2 were found in the nucleus in both FC and FS cells, with perhaps higher basal levels seen in the FS cells (Fig. 5). However, after exposure to IR, the levels of MDM2 in the nucleus of FS cells decreased, before returning to basal levels by 24 h. In contrast, the level of MDM2 in FC cells rapidly increased by 3 h after IR and gradually returned to basal levels by 24 h. The levels of p21WAF1/cip1 in nuclear lysates were undetectable in both cell types.

The conclusion, from these observations, is that the regulation of p53 between the nucleus and cytoplasm is abnormal in cells deficient in DNA-PK activity, and the consequence of this abnormal regulation is impaired activation of downstream genes p21WAF1/cip1 and MDM2. Furthermore, the delayed and attenuated G1/S cell cycle checkpoint appears to be a consequence of the effect on p21WAF1/cip1. MDM2 appears to decrease from the nucleus after DNA damage when there is a lack of DNA-PK activity, but the opposite response is seen in normal cells.

DISCUSSION

The results in this paper suggest that, in scid cells, activation of downstream genes by p53, in response to DNA damage, is delayed and attenuated, and only by looking at a detailed time course is this effect seen for both induced protein levels and G1/S cell cycle arrest. They further suggest that, even without exogenous DNA damage, p53 is accumulating in the nucleus, but in a form that is less capable of triggering the downstream effects. Both of these observations are consistent with the hypothesis that DNA-PK is modifying p53 function,
Fig. 4. Immunofluorescence of FC and FS cells, stained with pAb421, without (left) and with (right) IR exposure. Spindle-shaped fibroblasts show predominantly cytoplasmic p53 in FC cells (upper panel) prior to irradiation, and a predominantly nuclear staining pattern after IR. FS cells (lower panel) show a predominantly nuclear staining pattern with or without IR.

Fig. 5. Immunoblot of nuclear lysates, probed with 2A10 antibody for MDM2. Time points are immediately prior to irradiation and 3, 6, 9, and 24 h after irradiation. Thirty micrograms of protein were loaded per lane. Separate panels for FS (upper) and FC (lower); positive control is SAOS-2 transfected with pCMV-MDM2, a derivative of pCMV-SN3 (66), with human MDM2 cloned into the BamHI sites. An arrow marks the position of MDM2.

directly or indirectly, without affecting the ability of cells to accumulate p53 protein.

Altered p53-dependent DNA Damage Response in Scid Cells—The signaling pathway regulating the degradation of p53 in response to DNA damage, which is thought to be the main mechanism regulating the levels of p53 following DNA damage (56, 57), is not critically dependent upon DNA-PK. Wei and others (34, 35, 38) have found that the levels of p53 rise and fall in scid mouse derived MEF just as in normal MEF. However, the ability of p53 to activate downstream genes appears to be, at least in part, dependent on DNA-PK, consistent with and complementary to the findings of Woo et al. (39). A link between DNA-PK and p53, using an in vivo response, has not previously been reported. The justification for this conclusion is the kinetics of the induction of the downstream genes p21WAF1/cip1 and MDM2, and the kinetics of G1/S arrest. The distribution of p53 between the cytoplasm and nucleus appears to be the major defect: abnormally high levels of p53 are seen in the nucleus in scid cells but this p53 is in a form that is relatively inactive and less able to activate downstream genes.

This abnormal G1/S arrest appears to be in contradistinction to recent reports for MEF (34, 38), which demonstrated that the G1/S arrest in response to DNA damage in scid cells was equivalent in magnitude to that of the wild-type parental line. However, the report by Huang et al. (34) shows G1/S arrest profiles only at 24 h, and at that time point, little difference between normal and scid cells was observed in our studies (see Fig. 1). The report by Rathmell et al. (38) shows flow cytometric profiles with 5-bromodeoxyuridine antibody analysis at 6–8 h following 8 Gy IR in MEF, which appears to be equivalent to the analysis presented in this report. However, it is clear that both normal and scid cells have a significant tetraploid population, which is a sign of genetic instability (58). In our unpublished observations with MEF, when tetraploidy is observed, loss of p53 function follows within a small number of further passages in culture. As a consequence, MEF showing tetraploidy are rejected for further use and analysis.

Cells deficient in DNA-PK activity by mutation or knockout of Ku 80 have been reported to have a normal G1/S checkpoint at time points less than 12 h (59). It is not clear whether this reflects functional differences between scid, which has mutant DNA-PKcs, and Ku 80 null cells. A DNA-PKcs-null mouse has recently been established (60, 61) but p53 responses have not yet been reported for comparison. It was of considerable interest that the DNA-PKcs null mouse and the MEF cells derived from the mouse showed a phenotype that was identical to the scid mouse and scid cells, in all aspects in relation to DNA repair and VDJ recombination, suggesting that the phenotype of scid is much closer to the null phenotype, despite the residual detectable DNA-PKcs protein and DNA-PK activity in the in vitro assay (39). The relationship between the measured DNA-PK activity in vitro and the phenotype seen in vivo may not correlate as well as predicted by purely in vitro studies. At the time of our studies, there was no alternative DNA-PKcs deficient line to study for comparison. The hamster line, V3 (62), which does have a mutation in DNA-PKcs, has inactivated p53.

It is only by analyzing cell cycle profiles, induced levels of p21WAF1/cip1 and MDM2 at every 3 h, that the differences between scid cell and normal cells becomes apparent. Rathmell et al. (38) show a Northern blot analysis of p21 mRNA, at a single time point 4 h after exposure to IR, and report equivalent induction. A single time point could reflect a decreasing value for normal cells and an increasing value for scid cells. No protein analysis, in conjunction with the mRNA studies, was shown for p21, perhaps because the levels are extremely low (as we observed). Analysis of cells with p53 function eliminated would show that the p21 induction was p53-dependent (63). The elevation of p21 and MDM2 levels beyond 12 h shown in this report suggests that their activation by p53 is delayed and attenuated. One feature of scid cells is that the levels of p53 peak again at 24 h, and this is presumably due to unrepaired damage continuing to maintain the signal to inhibit degradation.

It is clear that residual p53 function is present in scid cells, as elimination of p53 either by knockout or by E6 transfection results in complete loss of the G1/S checkpoint and a failure to induce p21 or MDM2.

DNA-PK, p53, and MDM2—Regulation of the function of p53 as a transcription factor is dependent on an autoregulatory feedback loop with MDM2 (5). It has been shown that DNA-PK phosphorylates p53 in vitro at serines 15 and 17 (17), and that various types of DNA damage result in an increase of phosphorylated p53 at serine 15 (18). When p53 is phosphorylated at this location, its ability to transactivate is improved and its interaction with MDM2 is inhibited. In principle, this would appear to predict a model in which deficient DNA-PK activity results in unphosphorylated p53 at serines 15 and 37, which in turn would result in both decreased immediate transactivation and slower stimulation of downstream genes. These predictions are entirely in keeping with our observations. In addition, Fiscella et al. (20) reported that a cell line containing a serine 15 phosphorylation mutant of p53 had a delayed G1/S checkpoint. However, Shieh et al. (18) also report (without showing specific data) that p53 is constitutively phosphorylated at serine 15 in scid cells, which is not the prediction of the model. It may be that focusing on one phosphorylation site is an oversimplification of the constitutive regulation of p53 and its regulated function in the cell cycle.
response to DNA damage. The modification of other sites within p53 may affect the impact of phosphorylation in the N-terminal domain. Another possibility is that MDM2 is also phosphorylated by DNA-PK, and regulation of MDM2 function is also controlled by phosphorylation or dephosphorylation. The amino acid sequence of MDM2 does predict potential substrate sites for DNA-PK.

**Alterations of p53 in scid without DNA Damage: Nuclear Cytoplasmic Distribution**—Further characterization of the activation of p53 and the transactivation of downstream genes has revealed abnormalities in the localization of p53 between the cytoplasm and nucleus, from both immunoblotting and immunofluorescence. Curiously, there were high levels of p53 in the nucleus of *scid* cells prior to any exposure to IR, whereas normal cells maintain p53 predominantly within the cytoplasm until DNA damage, when it promptly moves to the nucleus (43). Why should there be abnormally high levels of p53 in the nucleus in *scid* cells prior to irradiation? It is known that many types of mutant p53 can accumulate in cells, with a predilection for the nucleus, but in *scid* cells it was shown that partial p53 function is preserved. Perhaps the accumulation of p53 into the nucleus is a reflection of a repair deficient cell, which has chronically accumulated DNA damage, which in turn maintains a damage signal to p53. If there were a physiologic accumulation of p53, then transactivation should not be impaired and the cell should either be growth arrested or ready to arrest with little delay.

Loss of DNA-PK activity in *scid* cells results in a loss normal maintenance of p53, as well as an attenuated and delayed DNA damage response. We expect the DNA-dependent protein kinase to be a DNA damage-dependent protein kinase, but activity of the kinase can be detected in the absence of exogenous DNA damage and in a cell cycle-dependent manner, with peaks in G1 and G2 (64). It would clearly be undesirable to respond to the DNA breaks in S-phase as if they were exogenous DNA damage, and part of normal maintenance may keep p53 in the DNA breaks in S-phase as if they were exogenous DNA damage, and part of normal maintenance may keep p53 in the nucleus of *scid* cells to a repair deficient cell, which has chronically accumulated DNA damage, which in turn maintains a damage signal to p53. If there were a physiologic accumulation of p53, then transactivation should not be impaired and the cell should either be growth arrested or ready to arrest with little delay.

The phosphorylation state of a protein is the predominant mechanism for regulating transport between the cytoplasm and nucleus. Although p53 accumulates in the nucleus of *scid* cells, it does not allow rapid transactivation, again implying that p53 in *scid* cells has a different post-translational modification from normal cells.

**The Functional Significance of Delayed Transactivation**—The loss of DNA-PK activity in *scid* cells results in decreased double-strand break repair, as a consequence of impaired non-homologous recombination, and an apparent direct role of DNA-PK in DNA repair. It seems likely that DNA-PK may also play a role in sensing DNA damage, and as a protein kinase, function in a signal transduction pathway. What functional consequences occur as a result of delayed transactivation? It is not clear how this contributes to the phenotype of *scid* cells, whose defect seems to be largely explained by the impairment of the G1/S checkpoint. Cells deficient in p21<sup>WAF1/cip1</sup> have a partial loss of the G1/S checkpoint, also have no significant phenotype other than the checkpoint abnormality (65).

The delayed transactivation could potentially affect the activation of apoptotic pathways, but this has not been evaluated in this paper or other studies. MEF<sup>+</sup> are not a useful cell system to study apoptosis. DNA-PK mutations are not generally found in genetically unstable cells or in transformed or tumor cells. Therefore, although abnormalities of p53 activation have been detected in *scid* cells, the functional consequences of these abnormalities have not been established: it is clear that many functions of p53 remain intact within *scid* cells. It is likely that many signal pathways may contribute to post-translational modification of p53: loss of DNA-PK activity may cause an imbalance in the normal regulatory process. The combination of delayed G1/S checkpoint, abnormal p21<sup>WAF1/cip1</sup>, and MDM2 induction, and abnormal nuclear localization of p53 and MDM2 provide compelling evidence that DNA-PK activity does affect p53 function. The additional recent evidence about the effect of DNA damage, resulting in phosphorylation of serines 15 and 37, activated DNA binding and increased transactivation in a reporter assay supports this view. The signal pathways from DNA damage to p53 appear to be distinct between post-translational modification of p53 (which is DNA-PK dependent) and degradation of p53 (which thus far appears independent of DNA-PK).

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