DNA-PK and Chk2 synergistically activate a latent population of p53 upon DNA damage

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Running title: p53 activation by DNA-PK and Chk2

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JBC Papers in Press. Published on January 29, 2004 as Manuscript M309917200
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Summary

The role of the checkpoint kinase 2 (Chk2) as an upstream activator of p53 following DNA damage has been controversial. We have recently shown that Chk2 and the DNA-dependent protein kinase (DNA-PK) are both involved in DNA damage-induced apoptosis but not G1 arrest in mouse embryo fibroblasts (MEFs). Here we demonstrate that Chk2 is required to activate p53 in vitro as measured by its ability to bind its consensus DNA target sequence following DNA damage, and is in fact the previously unidentified factor working synergistically with DNA-PK to activate p53. The gene mutated in ataxia telangiectasia (ATM) is not involved in this p53 activation. Using wortmannin, serine 15 mutants of p53, DNA-PK null cells and Chk2 null cells, we demonstrate that DNA-PK and Chk2 act independently and sequentially on p53. Furthermore, the p53 target of these two kinases represents a latent (pre-existing) population of p53. Taken together, the results from these studies are consistent with a model in which DNA damage causes an immediate and sequential modification of latent p53 by DNA-PK and Chk2, which under appropriate conditions, can lead to apoptosis.
Introduction

In response to DNA damage induced by ionizing radiation (IR), eukaryotic cells can activate cell cycle checkpoints or apoptosis. The p53 tumour suppressor mediates these cell responses (1-4), however, understanding of the mechanism of its activation remains elusive. Upstream candidates include the gene mutated in Ataxia telangiectasia (ATM) (5,6), the DNA dependent kinase catalytic subunit (DNA-PKcs) (7-9), and more recently the evolutionarily conserved checkpoint kinases Chk1 and Chk2 (10-15).

Chk2 has been demonstrated to form stable complexes with p53 (16) and was proposed to activate p53 via its kinase activity toward Ser20 on p53 (10,11). Further, Chk2 has been identified in a subset of Li Fraumeni patients with normal p53 alleles making it a potential tumour suppressor protein (17-19). How endogenous Chk2 functions in this capacity has been the focus of many recent studies. Chk2-/- murine embryonic stem (ES) cells have been examined and reported to be unable to maintain IR-induced G2 arrest and murine Chk2-/- thymocytes and mouse embryo fibroblasts (MEFs) have been shown to have an attenuated apoptotic response (12). The role of Chk2 in the G1 cell cycle checkpoint in the murine model has been controversial with some groups reporting its requirement while others reporting it as unnecessary for the p53 mediated G1/S arrest (12-15).

The role of Chk2 in the human p53 responses has recently been questioned. Ahn et al. (2003) (20) have purified Chk2 from DNA damaged human cells and demonstrated that p53 phosphorylation is not enhanced after IR. Furthermore, following introduction of Chk2 short
interfering RNA (siRNA) into three different human tumour cell lines, p53 was still found to be stabilized and active after IR. Concurrently, Jallepalli et al. (2003) (21) disrupted the Chk2 gene in human cancer cells and found that p53 Ser20 phosphorylation, stabilization, transcriptional activation as well as its cell cycle mediated arrest and apoptotic responses remained intact. These two reports call into question the role of Chk2 in human cells and further question whether Chk2 lies upstream of the p53-dependent apoptotic response as it seems clear it does in the murine system. More recently however, Craig et al. (22) have demonstrated that Chk2 is regulated by allosteric effects of p53 and that its kinase-dependent phosphorylation of p53 requires conformational docking sites on p53, lending evidence again to a role for human p53 as a target of Chk2 phosphorylation.

As disruption of the p53 tumour suppressor is often found in many human cancers, understanding the mechanism of p53 activation is essential to our progress in the treatment of cancer. We have recently reported that DNA-PK and Chk2 are both required for p53-mediated apoptosis in MEFs and that they function in a pathway that uses latent p53 to mediate this response (8,13). In the present study we used various approaches to establish the link between these two kinases in p53 activation following DNA damage. We show that Chk2 and DNA-PK act synergistically and in parallel to activate p53 (including human p53) as measured by its ability to bind its consensus DNA target sequence. The p53 target of these two kinases is a latent (pre-existing) population of p53. The gene mutated in ataxia telangiectasia (ATM) is not involved in this p53 activation. Our results are consistent with a model in which the immediate actions of DNA-PK and Chk2 on latent p53 following DNA damage are the first events that dictate the subsequent cellular apoptotic response.
Experimental Procedures

Cell culture and preparation of nuclear extracts.

The two glioma cell lines M059K and M059J were grown in DMEM-F12 supplemented with 10% fetal bovine serum (GIBCO-BRL). A-T lymphoblasts were obtained from ATCC and were cultured in F-12 DMEM with 20% fetal bovine serum (GIBCO-BRL). DNA-PK -/- or Chk2 -/- mouse embryo fibroblasts were isolated from 12.5-day-old embryos and were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were grown to 80% confluency and were then irradiated with a $^{137}$Cs irradiator at a rate of 2.5 Gy min$^{-1}$ for 2 min. Nuclear extracts were prepared as described (13).

Cell-free in vitro translation.

For cell-free in vitro translation, cytoplasmic extracts from M059K and M059J cells were prepared as reported (30), except that the extracts were not treated with micrococcal nuclease. Wild-type and mutants of human p53 mRNA were synthesized by in vitro transcription using a T7 polymerase Megascript kit (Ambion) and human Chk2 mRNA was synthesized using the T7 polymerase kit and the vector containing wild type human Chk2 sequence kindly provided by Dr. T. Halazonetis (Wistar Institute). The human Chk1 clone was a kind gift from Dr. Y. Sanchez (University of Cincinnati). In vitro translation reactions were carried out in a final volume of 50 µl containing the following components: 30 µl cytoplasmic extract, 1 mM ATP, 0.5 mM GTP, 1 mg ml$^{-1}$ creatine phosphokinase (from ICN), 10 mM creatine phosphate, 40 mM haemin (from ICN), 80 mM KCl, 5 mM magnesium acetate, 1 mM DTT, 5 µM of each amino acid (Promega), placental RNase inhibitor (RNA Guard, Pharmacia) and either water (for mock
translations) or p53 mRNA (for p53 translations). Translation reactions were incubated at 37°C for 30 min, followed by the addition of an equal volume of nuclear extract from mock-treated or γ-irradiated M059K or M059J cells (see above) that had been immunodepleted of endogenous p53 using the anti-p53 monoclonal antibody pAb421 preadsorbed onto inactivated Staph. A (IgSorb, The Enzyme Center). Reaction mixtures were then subjected to EMSA as described.

**DNA-binding analysis by EMSA.**

DNA binding was analysed by electrophoretic mobility shift assay (EMSA) using the ²³P-labelled p53 consensus sequence 5'AGCTTAGACATGCCTAGACATGCCAAGCT' as described (31) except that the binding reaction contained 5 mM DTT, 50 µM ZnCl₂, and 500 ng nonspecific oligonucleotides instead of salmon testis DNA, and samples were analyzed on a Tris–borate–EDTA polyacrylamide gel.

**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; 5%; 0.01% bromophenol blue). Electrophoresis was carried out at room temperature, with an applied current of 35 mA for approximately 3 h. Proteins were transferred to nitrocellulose for 2 h at 80 V, 4°C. The blot was then rinsed in TBS plus 0.2% Tween 20 (TBS-T), and placed in blocking buffer with 5% non-fat milk powder in TBS-T overnight. Next, the blot was incubated in primary antibody, [FL393 (Santa Cruz), p53 S15 phospho-specific antibody and Chk2 Thr-68 phospho-specific antibody (Cell Signalling Technology)] at a dilution of 1: 1000 in blocking buffer for 1 h. Following incubation with the primary antibody, the blot was thoroughly washed in blocking buffer. Anti-rabbit (Jackson Labs)
IgG-HRP secondary antibody was used at 1:5000 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) and then exposed to Kodak X-OMAT film. For protein loading control, a monoclonal antibody to actin or tubulin (Sigma) was used at 1:10000 dilution and processing was carried out as described.
Results

DNA-PK and Chk2 are both required to activate p53 DNA binding in vitro

We have previously demonstrated that DNA-PK is necessary but not sufficient to activate p53 DNA binding in vitro, and that an unknown factor present in DNA damaged cells is also required for p53 activation (7). More recently, we reported that both DNA-PK and Chk2 are involved in p53-dependent apoptosis, and that the latent, rather than the inducible population of p53 is implicated in this process (8,13). To determine if the unknown factor that collaborated with DNA-PK in activating p53 in vitro was Chk2, we employed the same cell free translation system used previously to show that DNA-PK acts upstream of p53 in promoting p53 sequence specific binding (7). Accordingly, wild type human p53 was translated in a cytoplasmic extract prepared from the human glioma cell line M059J which lacks any DNA-PK activity (due to defective expression of DNA-PKcs) (23). Nuclear extract from either DNA-PK positive (M059K) or null (M059J) glioma cell lines (untreated or γ-irradiated) was then added to test whether the translated p53 could be activated for p53 binding. As previously reported, the irradiated glioma line with DNA-PK (M059K) activated p53 binding as did the glioma line lacking DNA-PK (M059J) if supplemented with purified DNA-PK (Figure 1, lanes 1-4). Most importantly, whereas DNA-PK or Chk2 (in vitro translated) alone was unable to activate p53 (lanes 5 & 6), together they activated p53 binding to the same extent as with the irradiated nuclear extract (lane 7). The specificity of the Chk2 kinase in this role was confirmed as Chk1 kinase did not cooperate with DNA-PK to activate p53 (data not shown). The ability of DNA-PK to activate p53 in the absence of DNA damage in this case can be explained by the fact that DNA-PK is activated by the presence of DNA ends supplied by the consensus DNA sequence.
As well, recently it has been shown that over-expression of Chk2 can result in auto- and trans-phosphorylation events that activate Chk2 in the absence of IR (24). Our experiment therefore identifies the previously hypothesized “unknown” nuclear factor acting with DNA-PK to activate p53 DNA binding \textit{in vitro} (13) as the checkpoint kinase Chk2. In addition, since all the components involved are human in origin, it supports a possible role of Chk2 upstream of human p53.

\textbf{ATM is not required for activation of p53 sequence specific binding \textit{in vitro}}

We previously reported that whereas both DNA-PK and Chk2 are required for activating latent p53 mediated apoptosis in mouse embryo fibroblasts (MEFs), ATM is dispensable for this activity (8,13). We now sought to determine if the \textit{in vitro} system required ATM to activate p53 DNA binding and establish if this system reflects the observed apoptotic situation in MEFs.

To this end, nuclear cell extracts were prepared from an ataxia telangiectasia (A-T) lymphoblastoid cell line and examined for their ability to activate p53 DNA binding in the cell free translation system. The p53 in these A-T cells is highly unstable and therefore no endogenous p53 protein was detectable by EMSA (Figure 2, lane 6 & 7) or by western blot (not shown). However, nuclear extracts from IR-treated A-T cells, like those from IR-treated M059K cells, were able to activate \textit{in vitro}-translated p53 (Figure 2, lanes 2-5). This suggests that ATM, while necessary for the stabilization of p53, is not required for the activation of p53 DNA binding. In an independent experiment, A-T cells were treated with leptomycin B to determine if accumulation of p53 by stabilization alone was enough to activate p53 DNA binding, and it was
found that it did not (data not shown). This is consistent with our previous results demonstrating that Chk2 functions independently of ATM to activate the p53 apoptotic response (13).

**DNA-PK and Chk2 act independently and sequentially to activate p53**

We then proceeded to determine whether DNA-PK and Chk2 act independently of one another or whether they are required for each other’s action. A number of experimental approaches were taken to examine the sequence of events in the activation of p53 by DNA-PK and Chk2.

First, using the *in vitro* system the fungal metabolite wortmannin was employed to inhibit DNA-PK before and after addition of various components in the pathway. As can be seen in Figure 3A, DNA-PK activity is required to activate p53 as inhibition of DNA-PK prior to its incubation with p53 prevented p53 DNA binding (compare lanes 3 and 4). By contrast, if DNA-PK and p53 are allowed to interact and then DNA-PK is inhibited by wortmannin prior to Chk2 addition, p53 binding can still be observed (Figure 3A, lane 5). This suggests that DNA-PK acts directly on p53 but it is not upstream of Chk2.

We have previously demonstrated that Ser15 on latent p53 is a target of DNA-PK immediately following DNA damage, and that this residue plays a role in IR-induced apoptosis of MEFs (8). To determine if Ser15 is also involved in the *in vitro* activation of p53 by DNA-PK, two ser15 mutants of p53, Ser15Ala (S15A) and Ser15Asp (S15D), were tested in our *in vitro* activation assays. Since S15D mimics phospho-Ser15, it was expected to be active even in the absence of DNA-PK, whereas S15A would be innately inactive. Unexpectedly, we found that for
both mutants, Chk2 was absolutely required, but DNA-PK was dispensable for activation of DNA binding (Figure 3B). This suggests that conformational changes in p53 brought on by Ser15 phosphorylation, rather than a difference in charge, are responsible for DNA-PK-induced activation of p53. The observation that for both S15A and S15D mutants, Chk2 alone was able to activate DNA binding in the absence of DNA-PK also strongly argues for Chk2’s independence from DNA-PK. It further implies that upon DNA damage, p53 is likely first acted on by DNA-PK before being further modified by Chk2.

We also altered a few other serine phosphorylation sites on p53 to see if these mutations would affect p53 activation by DNA-PK and Chk2 in any way. These included two N-terminal phosphorylation sites, Ser20 (a known target site of Chk2), and Ser37 (another known DNA-PK target site), and two C-terminal phosphorylation sites, Ser315 and Ser392. None of these mutations was found to alleviate the dependency on DNA-PK or Chk2 for p53 activation (Figure 3C). It is also interesting that mutation of p53 at Ser20, a known Chk2 target site, to either alanine (simulating the unphosphorylated state) or aspartic acid (simulating constitutive phosphorylation) had no effect on its requirement for Chk2, which in turn suggests that Ser20 is not the target of Chk2 for p53 activation in vitro.

That DNA-PK and Chk2 likely act on p53 independently of each other was further suggested by the following observations. First, Chk2+/+ and Chk2-/- MEFs displayed similar levels of DNA-PK-dependent Ser15 phosphorylation upon IR (Figure 3D). Second, Chk2 phosphorylation at threonine 68 (Thr68), often used as a measure of Chk2 activation (25-28), was found to be immediate and at comparable levels between human cells with and without DNA-PK (M059K and M059J, respectively) upon γ-irradiation (Figure 3E), These observations,
combined with those from the wortmannin and S15A and S15D studies described above (Figures 3A and 3B, respectively), have led us to conclude that DNA-PK and Chk2 work sequentially and independently of one another in activating p53 upon DNA damage.

**Latent but not inducible p53 requires Chk2 and DNA-PK for *in vitro* DNA binding**

We have recently reported that the latent population of p53 is sufficient to mediate the Chk2 and DNA-PK-directed p53 apoptotic response (7,13). To determine if it is the latent population of p53 whose DNA binding activity is regulated by DNA-PK and Chk2, wild type, Chk2 null, and DNA-PK null MEFs were exposed to IR in the presence and absence of cycloheximide. Treatment of the cells with cycloheximide blocks translation of any new p53 protein and allows us to assess the DNA binding activity of pre-existing (latent) p53. Figure 4 clearly shows that the latent p53 population in wild type MEFs are activated to bind DNA upon IR. In contrast, only the induced, but not the latent population of p53 was activated in DNA-PK-/- cells. This is consistent with our previous contention that DNA-PK targets latent p53, whereas ATM targets induced p53 (8,13).

Likewise, examination of wild type versus Chk2-/- cells yielded the same results, suggesting that Chk2 is absolutely required for latent p53 binding to its consensus sequence (Figure 4).
Discussion

Following DNA damage such as ionizing radiation, cell signalling events cause cell cycle arrest or apoptosis. The p53 tumour suppressor is central to these responses and has been shown to be a target of a number of kinases including ATM, ATR, DNA-PK, Chk1 and Chk2. Until recently, it has been strongly suggested that following IR, the Chk2 kinase was an upstream mediator of p53 cell cycle and apoptotic responses. With the more recent evidence questioning the role of Chk2 in human cancer cell lines it is important to decipher the role, if any, Chk2 plays in activating p53 responses.

To examine the role of Chk2 upstream of p53 we utilized an in vitro cell free translation system whereby p53 activation could be assessed by its ability to bind its consensus sequence. This system was previously used to demonstrate that DNA-PK acts upstream of p53 in response to DNA damage, and that an unknown factor (also activated by DNA damage) was also involved in this process. We now show that this unknown factor is Chk2. These two kinases work synergistically to activate p53 DNA binding that is demonstrable in vitro. Whether the p53 binding is an actual indication of p53 transcriptional activity or simply a readout for some conformation change in the p53 protein induced by Chk2 and DNA-PK is unclear at present. What is clear, however, is that Chk2 and DNA-PK together are capable of activating p53, including human p53, in vitro.

The observation that human ATM cell extract was also capable of promoting p53 DNA binding is also consistent with our previous demonstration that Chk2 activates p53-mediated
apoptosis independently of the ATM protein. Here again the results indicate that while ATM is necessary to stabilize the p53 protein, the ability of Chk2 and DNA-PK to activate p53 does not require ATM. This also underscores the need to consider stabilization and activation of p53 as two separate events.

The independent nature of DNA-PK and Chk2 in activating p53 in vitro and the sequential order of their involvement were determined using a number of approaches. We used wortmannin to show that DNA-PK must act upon p53 since inhibition of its kinase activity completely abolished p53 binding. However, if DNA-PK was first allowed to interact with p53, the subsequent addition of wortmannin did not interfere with the activating effect of Chk2 that was added later. This indicates that DNA-PK is not required upstream of Chk2 to promote p53 activation. The demonstration that S15 is phosphorylated by DNA-PK equally well in Chk2+/+ and Chk2-/- cells further attests to the complete independence of action of these two kinases.

Further evidence for a parallel, and sequential mechanism of p53 activation by DNA-PK and Chk2 comes from the observation that the two p53 S15 mutants, S15A and S15D, do not require DNA-PK, and can be activated by Chk2 alone. While this result is expected for the S15D mutant which mimics phospho-S15, it came initially as a surprise in the case of the S15A mutant. The most logical explanation for both of these mutants being able to bypass the DNA-PK requirement is that modification of serine 15 by any means (phosphorylation or mutation) leads to an altered conformation of p53 which is recognizable by Chk2. Indeed, previous work by Shieh et al. (29) shows that phosphorylation at the serine 15 site by DNA-PK alters the tertiary structure of p53. Very recently, Craig et al. (22) demonstrated that two peptides derived from the
DNA binding domain of p53 could bind Chk2 and allosterically stimulate the phosphorylation of full-length p53. Based on our present findings, we contend that the reason why full-length p53 by itself cannot activate Chk2 is likely because these Chk2-docking sites on native p53 are cryptic and are exposed only after phosphorylation of S15 by DNA-PK. Thus the initial modification of p53 by DNA-PK is a prerequisite for its subsequent modification by Chk2, with the two events probably occurring quickly in tandem and resulting in full p53 activation.

Finally, our study suggests that it is the latent population of p53 that is activated by DNA-PK and Chk2 upon DNA damage. This is consistent with our previous observation that latent p53 becomes phosphorylated at serine 15 immediately upon IR treatment, and that latent p53, rather than inducible p53, is sufficient to induce apoptosis in a DNA-PK-dependent and Chk2-dependent manner. It therefore appears that the fate of the cell (cell growth arrest or apoptosis) is determined very early on, possibly immediately following DNA damage. Depending on the nature and extent of DNA damage, p53 that appears later on the scene (inducible p53) may then be modified by other kinases such as ATM or ATR, and may have a function that is distinct from that of the latent population of p53.

In summary, results from our investigation are consistent with a model wherein upon DNA damage, DNA-PK and Chk2 act synergistically, and sequentially (with DNA-PK followed by Chk2) on a latent population of p53 (Figure 5). The modified p53 manifests DNA binding activity in vitro, which may reflect the actual event in vivo or an altered conformational state that is unrelated to its DNA binding activity. In either case, the extent of such modifications on this
latent population of p53 probably dictates the subsequent course of action, and depending on the cell context, can lead to apoptosis.
References


Acknowledgements

We thank Dr. T. Halazonetis (Wistar Institute) for the Chk2 clone and Dr. Y. Sanchez (University of Cincinnati) for the Chk1 clone. This research is supported by the National Cancer Institute of Canada with funds from the Canadian Cancer Society (to P.W.K.L). M. J. was the recipient of a Studentship from the Alberta Cancer Board.
Figure Legends

Figure 1. Co-requirement of DNA-PK and Chk2 for activation of p53 DNA binding in vitro.

Wild type p53 was translated in cytoplasmic extracts from M059J cells. The following was then added to the reaction: lane 1, control (nothing added); lane 2, nuclear extract from γ-irradiated M059J (J*) cells; lane 3, same as lane 2 except purified DNA-PK holoenzyme was also added; lane 4, nuclear extract from γ-irradiated M059K (K*) cells; lane 5, purified DNA-PK holoenzyme; lane 6, Chk2 (in vitro-translated); and lane 7, purified DNA-PK holoenzyme and Chk2 in combination. Samples from these reactions were then tested for p53 sequence-specific DNA binding activity by EMSA.

Figure 2. ATM is dispensable for in vitro activation of p53 DNA binding.

Wild type 53 was synthesized in a cell-free translation system and nuclear extracts from non-DNA-damaged or γ-irradiated (IR) M059K cells or A-T lymphoblasts were added. The reaction mixtures were then assayed for p53 sequence-specific binding by EMSA.

Figure 3. DNA-PK and Chk2 act independently and sequentially to activate p53.

A. Effect of wortmannin on p53 activation by DNA-PK and Chk2. Wild type p53 was translated in vitro. The following was then added to the reaction: lane 1, control (nothing added); lane 2, Chk2 (in vitro translated); lane 3, Chk2 and purified DNA-PK holoenzyme in combination; lane 4, DNA-PK that had been inactivated by wortmannin for 10 min, followed by Chk2; lane 5, DNA-PK for 10 min, then wortmannin for 10 min, then Chk2. p53 DNA binding was then assayed by EMSA.
B. Chk2 is required but DNA-PK is dispensable for S15 p53 mutant DNA binding. S15A or S15D p53 mutants were translated and assayed for their requirement of Chk2 and DNA-PK to induce DNA binding to the consensus sequence as assayed by EMSA.

C. Comparing the serine 15 modification (S15A) to those at other serine phosphorylation sites (S315A, S392A, S37A, S20A, and S20D) in terms of requirement for DNA-PK and Chk2 for activation. S15A requires only Chk2 whereas wild type as well as all the other serine mutants of p53 require both DNA-PK and Chk2 for activation.

D. Comparing DNA damage-induced phosphorylation on serine 15 of p53 in Chk2+/+ and Chk2/- MEFs. Cells were exposed to γ-irradiation and harvested at various times thereafter. Following SDS-PAGE and transfer to nitrocellulose, the membrane was probed with an anti-phosphoserine 15 antibody or for total p53 protein.

E. Comparing DNA damage-induced phosphorylation on threonine 68 of Chk2 in M059K and M059J cells. Cells were exposed to γ-irradiation and harvested at various times thereafter. Following SDS-PAGE and transfer to nitrocellulose, the membrane was probed with an anti-phosphothreonine 68 antibody or for total Chk2 protein.

Figure 4. DNA-PK and Chk2 are required for activation of latent p53 DNA binding following DNA damage.

Wild type, DNA-PK-/-, or Chk2-/- MEFs were mock-treated (CTRL), γ-irradiated (IR), cycloheximide treated (CHX) or CHX treated followed by IR (CHR + IR). Nuclear extracts were then prepared and assayed for p53 DNA binding by EMSA.
Figure 5. Model for activation pathway of latent p53 and apoptosis.

Immediately following DNA damage, DNA-PK is activated by strand breaks and phosphorylates a latent (pre-existing) population of p53 on Ser15. This induces a conformational change in the p53 protein such that it is recognized by the Chk2 kinase which then phosphorylates (and activates) the latent p53. The severity and nature of DNA damage incurred dictates the extent of this latent p53 activation which, in many cell types, likely represents the first obligatory event that leads to apoptosis. Under circumstances where cell growth arrest or DNA repair is the preferred outcome, a separate pathway (not shown) involving inducible p53 and mediated by ATM, ATR or other kinases comes into play.
Jack et al

Figure 2

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p53-DNA complex
Figure 3

A. Translated p53

B. S15A  S15D

D. Chk2 +/+  Chk2 -/-

E. M059K  M059J
Jack et al

Figure 4

Wild type

DNA-PK -/-

Chk2 -/-

CTRL  IR  CHX  CHX + IR

p53-DNA complex
Chk2, but not Chk1, cooperates with DNA-PK in activation of p53 DNA binding in vitro.
Wild type p53 was translated in cytoplasmic extracts from M059J cells. Purified DNA-PK holoenzyme, and in vitro-translated Chk1 and Chk2 were then added in various combinations as shown. Samples from these reactions were then assayed for p53 sequence-specific DNA binding activity by EMSA. [Inset: In vitro translation of Chk1 and Chk2 (35S-methionine labeling) showing that the two proteins were synthesized at comparable levels.]
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*J. Biol. Chem.* published online January 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309917200

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