DNA-dependent Protein Kinase and Checkpoint Kinase 2
Synergistically Activate a Latent Population of p53 upon
DNA Damage*

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Melissa T. Jack§, Richard A. Woo‡, Noboru Motoyama‡, Hitoyuki Takaï¶,
and Patrick W. K. Lee‡

From the §Cancer Biology Research Group and Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre Calgary, Alberta T2N 4N1, Canada and the ¶Department of Geriatric Research, National Institute for Longevity Sciences, Oba, Aichi 474-8522, Japan

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 embryonic fibroblasts. 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 in fact the previously unidentified factor working synergistically with DNA-PK to activate p53. The gene mutated in ataxia telangiectasia 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 (preexisting) 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.

In response to DNA damage induced by ionizing radiation (IR),1 eukaryotic cells can activate cell cycle checkpoints or apoptosis. The p53 tumor suppressor mediates these cell responses (1–4); however, an understanding of the mechanism of its activation remains elusive. Upstream candidates include the gene mutated in ataxia telangiectasia (ATM) (5, 6), the DNA-dependent protein kinase (DNA-PK) catalytic subunit (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 Ser-20 on p53 (10, 11). Furthermore, Chk2 has been identified in a subset of Li Fraumeni patients with normal p53 alleles, making it a potential tumor suppressor protein (17–19). How endogenous Chk2 functions in this capacity has been the focus of many recent studies. Chk2 is required for p53-mediated apoptosis upon DNA damage and checkpoint activation by IR in mammalian cells (18, 20). This work supports the idea that Chk2 is a mediator of DNA damage-induced G1 arrest and apoptosis.

The role of Chk2 in the human p53 responses has recently been questioned. Ahn et al. (20) have purified Chk2 from DNA damaged human cells and demonstrate that p53 phosphorylation is not enhanced after IR. Furthermore, following introduction of Chk2 short interfering RNA into three different human tumor cell lines, p53 was still found to be stabilized and active after IR. Concurrently, Jallepalli et al. (21) disrupted the Chk2 gene in human cancer cells and found that p53 Ser-20 phosphorylation, stabilization, and 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 because it seems clear that it does lie upstream 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.

Because disruption of the p53 tumor 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 this 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 (preexisting) population of p53. The gene mutated in 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.

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¶ To whom correspondence should be addressed: Dept. of Microbiology and Immunology, 7-P, Sir Charles Tupper Medical Bldg, Dalhousie University, 5850 College St., Halifax, Nova Scotia B3H 1X5, Canada. Tel.: 902-494-8048; Fax: 902-494-5125; E-mail: patrick.lee@dal.ca.

1 The abbreviations used are: IR, ionizing radiation; ATM, ataxia telangiectasia-mutated; DNA-PK, DNA-dependent protein kinase; Chk, checkpoint kinase; MEF, mouse embryonic fibroblast; EMSA, electrophoretic mobility shift assay.
EXPERIMENTAL PROCEDURES

Cell Culture and Preparation of Nuclear Extracts—The two glioma cell lines, M059K and M059J were grown in Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum (FBS). A-T lymphoblasts were obtained from ATCC and were cultured in Dulbecco’s modified Eagle’s medium/F-12 with 20% fetal bovine serum (FBS). DNA-PK−/− or Chk2−/− mouse embryo fibroblasts were isolated from 12.5-day-old embryos and were cultured in Dulbecco's modified Eagle’s medium supplemented with 10% fetal bovine serum.

Cells were grown to 80% confluency and were then irradiated with a 137Cs irradiator at a rate of 2.5 grays min⁻¹ for 2 min. Nuclear extracts were prepared as described previously (13).

Cell-free in Vitro Translation—For cell-free in vitro translation, cytoplasmic extracts from M059K and M059J were prepared as reported previously (7). 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 of cytoplasmic extract, 1 μl ATP, 0.5 μl GTP, 1 μl mg⁻¹ creatine phosphokinase (from ICN), 10 mM creatine phosphate, 40 mM hemin (from ICN), 80 mM KCl, 5 mM magnesium acetate, 1 mM dithiothreitol, 5

rabbit (Jackson Laboratories) IgG-horseradish peroxidase secondary antibody (Promega), placental RNase inhibitor (RNA Guard, Amersham Biosciences), 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 Staphylococcus A (IgSorb, The Enzyme Center). Reaction mixtures were then subjected to electrophoretic mobility shift assay (EMSA) as described.

DNA Binding Analysis by EMSA—DNA binding was analyzed by EMSA using the 32P-labeled p53 consensus sequence 5'-AGCTTTAGACTCATCCAGGATCCAGCT-3' as described previously (7).

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, 2% β-mercapto-ethanol, 0.01% bromophenol blue). Electrophoresis was carried out at room temperature with an applied current of 35 mA for ~3 h. Proteins were transferred to nitrocellulose membranes for 2 h at 80 V, 4 °C. The blot was then rinsed in Tris-buffered saline plus 0.2% Tween 20 and placed in blocking buffer with 5% nonfat milk powder in Tris-buffered saline plus 0.2% Tween 20 overnight. The blot next was incubated in primary antibody (FL393 (Santa Cruz Biotechnology), p53 Ser-15-phosphospecific antibody, and Chk2 Thr-68-phosphospecific antibody (Cell Signaling 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. Antibodies (Jackson Laboratories) IgG-horseradish peroxidase secondary antibody was used at 1:5000 dilution in blocking buffer and incubated at room temperature for 30–45 min followed by washing in blocking buffer. The blot was then subjected to chemiluminescence (ECL, Amersham Biosciences) and then exposed to Kodak X-Omat AR film.

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 whether 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 (because of defective expression of DNA-PK catalytic subunit) (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 (7), the irradiated glioma line with DNA-PK (M059K) activated p53 binding as did the glioma line lacking DNA-PK (M059J) if supplemented with purified p53 (Fig. 1, lanes 1–4). Most importantly, whereas DNA-PK or Chk2 (in vitro translated) alone was unable to activate p53 (lanes 5 and 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 because 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. Also, it has been shown recently that overexpression of Chk2 can result in autophosphorylation and trans-phosphorylation events that activate Chk2 in the absence of IR (24).

Therefore, our experiment identifies the previously hypothesized “unknown” nuclear factor acting with DNA-PK to activate p53 DNA binding in vitro (13) as the checkpoint kinase Chk2. In addition, because all of the components involved are human in origin, our experiment supports a possible role of Chk2 upstream of human p53.

ATM Is Not Required for Activation of p53 Sequence-specific Binding in Vitro—We previously reported that whereas both DNA-PK and Chk2 are required for activating latent p53-mediated apoptosis in MEFs, ATM is dispensable for this activity (8, 13). We now seek to determine whether the in vitro system required ATM to activate p53 DNA binding and establish whether 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 (Fig. 2, lanes 6 and 7) or by Western blot (data not shown). However, nuclear extracts from IR-treated A-T cells, similar to those from IR-treated M059K cells, were able to activate in vitro translated p53 (Fig. 2, lanes 2–5). This finding suggests that ATM, although 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 whether accumulation of p53 by stabilization alone was enough to activate p53 DNA binding and it was found that it was not (data not shown). This finding 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.

Using the in vitro system, the fungal metabolite wortmannin was employed to inhibit DNA-PK before and after the addition of various components in the pathway. As can be seen in Fig. 3A, DNA-PK activity is required to activate p53 because 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 (Fig. 3A, lane 5). This finding suggests that DNA-PK acts directly on p53 but it is not upstream of Chk2.

We have previously demonstrated that Ser-15 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 whether Ser-15 is also involved in the in vitro activation of p53 by DNA-PK, two Ser-15 mutants of p53, S15A and S15D, were tested in our in vitro activation assays. Because S15D mimics phospho-Ser-15, 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 (Fig. 3B). This finding suggests that conformational changes in p53 brought on by Ser-15 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 the independence of Chk2 from DNA-PK. It further implies that upon DNA damage, p53 is probably 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 whether these mutations would affect p53 activation by DNA-PK and Chk2 in any way. These included two N-terminal phosphorylation sites, Ser-20 (a known target site of Chk2) and Ser-37 (another known DNA-PK target site), and two C-terminal phosphorylation sites, Ser-315 and Ser-392. None of these mutations was found to alleviate the dependence on DNA-PK or Chk2 for p53 activation (Fig. 3C). It is also interesting that the mutation of p53 at Ser-20, 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 Ser-20 is not the target of Chk2 for p53 activation in vitro.

That DNA-PK and Chk2 probably 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 Ser-15 phosphorylation upon IR (Fig. 3D). Second, Chk2 phosphorylation at Thr-68, 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 (Fig. 3E). These observations, combined with those from the wortmannin and S15A and S15D studies described above (Fig. 3, A and B, 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 whether 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 preexisting (latent) p53. Fig. 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 finding is consistent with our previous contention that DNA-PK targets latent p53, whereas ATM targets induced p53 (8, 13). Likewise, an 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 (Fig. 4).

**DISCUSSION**

Following DNA damage such as ionizing radiation, cell signaling events cause cell cycle arrest or apoptosis. The p53 tumor 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, that 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 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 required for activation of latent p53 following DNA damage.

FIG. 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, wortmannin for 10 min, and then Chk2. p53 DNA binding was then assayed by EMSA. B, Chk2 is required, but DNA-PK is dispensable for Ser-15 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 of 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 membrane, 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 membrane, the membrane was probed with an anti-phosphothreonine 68 antibody or for total Chk2 protein.

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 required for activation of latent p53 following DNA damage.
p53 Activation by DNA-PK and Chk2

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, although 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 Ser-15 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 Ser-15 mutants, S15A and S15D, do not require DNA-PK and can be activated by Chk2 alone. Although this result is expected for the S15D mutant, which mimics phospho-Ser-15, 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 probably because these Chk2-docking sites on native p53 are cryptic and are exposed only after phosphorylation of Ser-15 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 finding 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. Therefore, 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 (Fig. 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.

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