Cdc25C and p53 have been reported to be physiological targets of checkpoint kinase 2 (Chk2). Surprisingly, although Chk2 purified from DNA damage sustaining cells has dramatically increased ability to phosphorylate Cdc25C when compared with untreated cells, its ability to phosphorylate p53 is weak before treatment, and there is no increase in its activity toward p53 after DNA damage by γ irradiation or the radiomimetic agent nocodazol. Furthermore, introduction of Chk2 short interfering RNA into three different human tumor cell lines leads to marked reduction of Chk2 protein, but p53 is still stabilized and active after DNA damage. The results with Chk1 short interfering RNA indicate as well that Chk1 does not play a role in human p53 stabilization after DNA damage. Thus, Chk1 and Chk2 are unlikely to be regulators of p53 in at least some human tumor cells. We discuss our results in the context of previous findings demonstrating a requirement for Chk2 in p53 stabilization and activity.

The p53 tumor suppressor protein has been well studied as a target of numerous stress-induced signaling pathways in mammalian cells. Various forms of DNA damage bring about stabilization and activation of the p53 tumor suppressor protein presumably through prevention of the interaction of p53 with its negative regulator Mdm2. Induction of p53, a sequence-specific transcriptional activator, results in expression of a number of gene products whose function is to either arrest cell growth or promote apoptosis. As such, p53 has been defined as a checkpoint factor (reviewed in Refs. 1–3). ATM is a protein kinase with homology to members of the phosphatidylinositol 3-kinase family, and of the various DNA damage pathways to p53, that involving ATM has been the best documented; ATM-null cells fail to induce p53 after some forms of DNA damage (4), and the ability of ATM kinase to phosphorylate p53 is stimulated by specific types of DNA damage such as those caused by ionizing radiation or radiomimetic agents (5–7).

The ATM pathway itself is functionally related to DNA damage checkpoints in both budding and fission yeasts. In fission yeast, as yet unidentified DNA damage sensor proteins may signal directly or indirectly via downstream kinases or adaptor proteins to the homologous mediator kinases Rad3 and Tel1, which in turn regulate through phosphorylation two effector kinases Cds1 and Chk1 (8). A key target for these kinases is the Cdc25 dual specificity phosphatase that in unstressed cycling cells removes repressing phosphates from the cyclin-dependent kinase Cdc2 and thereby allows it to promote passage through G2/M (reviewed in Ref. 9). When the human homologues of Chk1 or Cds1 were identified and cloned, it became clear that they could phosphorylate different members of the human Cdc25 family (10–14). Phosphorylation of human Cdc25C at Ser216 leads to its inactivation by 14-3-3-mediated translocation to the cytoplasm (15, 16). There are a number of lines of evidence showing that in human cells ATM is upstream of Chk2. Perhaps the most compelling of these are observations that ATM phosphorylates Chk2 at Thr68 in vitro and that ionizing radiation leads to Chk2 phosphorylation at Thr68 and its subsequent activation in wild type but not ATM null cells (17–19).

The relationship between the ATM pathway and Chk1 is less well understood. It is currently believed that Chk1 kinase is downstream of ATR (ataxia-telangiectasia and Rad3-related kinase), another member of the phosphatidylinositol 3-kinase family (20–22). Although it is possible to generate cells and animals lacking ATM and Chk2, deletion of ATR or Chk1 causes early embryonic lethal events (20, 23, 24). More recently, homozygous deletion of Chk1 in the B cell lymphoma line, DT40, revealed that this kinase is essential for G2/M arrest and that its loss decreases survival after γ irradiation (25).

Upon various forms of cellular stress, p53 becomes phosphorylated at a number of sites within its N and C termini (reviewed in Ref. 26). Phosphorylation of p53 at N-terminal sites such as Ser15, Thr18, Ser20, and Ser37 within the vicinity of the regions where it interacts with Mdm2 can disrupt its interaction with Mdm2 in vitro (27–31) and may thereby allow for p53 stabilization (32, 33). Among the possible sites, Thr18 and Ser20 lie within the region of p53 that interacts directly with Mdm2 (34). Mutation of Ser20 in human p53 was shown to render p53 less well stabilized after DNA damage and more sensitive to down-regulation by Mdm2 (35–37). By contrast, murine fibroblasts harboring a mutation of the equivalent residue to human Ser20 that changes murine Ser23 to Ala23 are apparently normal in their response to DNA damage (38).

Given that both Chk1 and Chk2 are checkpoint effector kinases, it was not unexpected that several groups have linked them experimentally with p53. Using a biochemical fractionation approach to identify Ser20 kinase activity, we previously discovered that the human homologues of Chk1 and Chk2 could phosphorylate p53 at this site as well as a number of other sites within the N terminus such as Ser15, Thr18, and Ser37 along with unidentified sites within other regions of the
protein (39). Moreover, expression of either Chk1 kinase-defective or Chk1 antisense constructs leads to diminution of levels of co-transfected p53 protein (39). In a parallel study Chehab et al. (30) reported that Chk2 can phosphorylate Ser20 and that a kinase defective form of Chk2 prevents stabilization and phosphorylation at Ser20 of co-transfected p53 after DNA damage. Overexpression of Chk2 in U2OS cells, which carry wild type p53, augmented G1 arrest following irradiation. Further support for a role for Chk2 as being upstream of p53, Hirao et al. (40) reported that thymocytes and fibroblasts generated from Chk2 knock-out mice are defective in accumulating p53 after γ but not UV irradiation. Intriguingly, a second group using mouse embry-fibroblasts derived from these mice found that even at low doses of γ radiation, G1 arrest and p21 induction were intact (41). More recently, the results from a second independently generated Chk2 null mouse have indicated that Chk2 loss protects mice from γ irradiation-induced death consistent with diminished apoptosis in several tissues including the spleen, intestine, and central nervous system and that p53 in these cells is transcriptionally inactive (42).

Finally, data from human patients have revealed that a subset of Li-Fraumeni cancer-prone families with wild type p53 has Chk2 germ line mutations (43, 44), and such mutant forms of Chk2 are defective as protein kinases (45, 46). Strikingly, this natural experiment is not completely recapitulated in murine models. Hirao et al. (47) observed no increase in spontaneous tumors in Chk2−/− mice, whereas Takai et al. (42) reported preliminary evidence of an increase in lymphoma development by 71 weeks in Chk2−/− animals.

Given that there is somewhat contradictory evidence surrounding the proposed Chk2-p53 connection, we set out to further characterize Chk2 derived from human tumor cells that have an intact DNA damage response. The goal was to study Chk2 before and after activation by either γ irradiation or the radiomimetic compound necrocinostatin (NCS). To our surprise, the results from both biochemical and short interfering RNA (siRNA) experiments argue against a role for Chk2 in DNA damage-mediated stabilization of p53 in cancer cells following γ radiation or similar stimuli. We discuss the basis for the differences between our present results and those that have been previously published, including our own.

**EXPERIMENTAL PROCEDURES**

**Mammalian Cell Lines and Culture Conditions—**An HCT116-derived cell line stably expressing N-terminally HA-tagged wild type hChk2 (HA-Chk2) was kindly provided by Dr. J. Chen (Mayo Clinic, Rochester, MN) (45). These cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum. The cells were obtained from the American Type Culture Collection (Manassas, VA).

**Purification of Proteins—**Where indicated, the cells were treated with a radiomimetic compound NCS (500 ng/ml) (Kawaya Co., Tokyo, Japan) for 2 h before purification of HA-Chk2 as described below. Alternately, the cells were irradiated with 14 Gy (using a 137Cs source) and harvested after 2 h for purification of HA-Chk2. Typically, cells in 20 × 150-mm plates were collected and treated with lysis buffer A containing 50 mM Hepes KOH, pH 7.8, 150 mM NaCl, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, and 20% glycerol. C-terminally FLAG-tagged wild type and mutant (D347A) Chk2 (Chk2-FLAG) mutants were purified from baculovirus-infected insect cells as follows. The cells were harvested after lytic buffer A and the extracts were precipitated with 0.5 ml of glutathione-Sepharose 4B beads (Amersham Biosciences). GST-Chk2 proteins were eluted with 10 mM glutathione after washing away any unbound protein with lysis buffer A and then dialyzed as described above with buffer B.

The plasmid encoding GST-Chk2-FLAG (200–256) was kindly provided by Dr. Junjie Chen. GST fusion proteins, GST-Chk2-FLAG (200–256), GST-p53 (1–82), GST-p53 (97–363), and GST-p53 (WT) were expressed in Escherichia coli BL21 cells after induction with isopropyl-β-D-thiogalactoside for 2.5 h (final concentration, 1 mM) at 20 °C. The proteins were purified by binding to glutathione-Sepharose 4B beads in lysis buffer A followed by elution with 10 mM of glutathione and dialyzed as described above. His-p53 (WT) was purified as described by Zhou et al. (48).

**In Vitro Kinase Assays and Western Blotting—**Typically 5–20 ng of kinase was incubated with 0.1–4 μg of GST fusion protein substrates. The reaction mixtures were incubated at 30 °C for 30 min in 20 μl of Buffer C containing 20 mM Hepes KOH, pH 7.8, 100 mM KC1, 10 mM MgCl2, 1 mM DTT, 60 mM okadacy acid, 240 μM Cypermethrin, 1 mM NaF, 100 μM NaVO4, and 100 μM ATP supplemented with 1 μCi of [γ-32P]ATP. The reactions were terminated by adding 20 μl of SDS-PAGE sample loading buffer. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and subsequently identified and quantitated by immunoblotting with the appropriate antibodies. The radiolabeled proteins were visualized with autoradiography and quantitated using PhosphorImager (Molecular Dynamics). Phosphorylation sites of phosphorylated proteins were obtained as follows: anti-GST and anti-FLAG (M2) were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoprecipitation Kinase Assays—HCT 116 parental cells in 4 × 140-mm plates were collected and treated with lysis buffer B as described above. The extracts were incubated with 50 μl of agarose beads conjugated with protein A (Amersham Biosciences) and 2 μg of anti-Chk2 antibody (Santa Cruz Biotechnology) for 4 h at 4 °C. The beads were washed with buffer A (15 × 0.5 ml). The indicated amount of beads were washed with GST-fused substrates in kinase buffer C, and the data were analyzed as described above.

**Phosphorylation of p53 by DNA-PK—**GST-p53 (1–82) (10 μg) was incubated with DNA-PK (100 ng) in buffer containing 25 mM Hepes, pH 7.9, 50 mM KC1, 10 mM MgCl2, 20% glycerol, 1 mM DTT, 100 μM ATP, and 10 μg/ml of DNA fragments generated from HpaII-digested pBlueScript. Purified DNA-PK was a generous gift of D. Chan (Lawrence Berkeley National Laboratory, Berkeley, CA). The mixtures were incubated for 4 h at 30 °C. Phosphorylation at Ser15 was confirmed by separating the reaction mixture on 10% SDS-PAGE, transferring to nitrocellulose, and immunoblotting with anti-Serp15-specific antibody (Cell Signaling, Beverly, MA). The phosphorylated GST-p53 was purified by binding to glutathione-Sepharose 4CL beads as described above.

**RNA Interference—**siRNA duplexes were synthesized by Xenogen Oligoribonucleotides (Huntsville, AL). The luciferase control sequence has been described previously (49). The sequences of the Chk1 oligonucleotides were: 5′-GAAAAGUCUCCAGUGAAATT-3′ and 5′-UC-UUCAGCGGAGCUCGUU-3′. The sequences of the Chk2 oligonucleotides were: 5′-GAAAAGUCUCCAGUGAAAC-3′ and 5′-GUUC-GAGCACUGUGAUGUGG-3′. The cells were transfected with 1 μg of each plasmid per 30-mm culture dish at 70% confluence. Chk1 was detected with a mouse monoclonal antibody (Santa Cruz Biotechnology), and Chk2 was detected with a rabbit polyclonal antibody (ProSci Inc., Poway, CA). p53 was detected using a mixture of monoclonal antibodies DO-1 and 1801. Protein loading was esti-
Fig. 1. DNA damage activates Chk2 to phosphorylate a fragment of Cdc25C but not the N terminus of p53. A, total cell extracts (100 μg) of HCT116 (HA-Chk2) (first lane) or HCT116 parental (second lane) cells were subjected to SDS-PAGE and then immunoblotted with anti-Chk2 antibody. B, HA-Chk2 was immunopurified from extracts of HCT116-HAChk2 cells (20 × 140-mm plates) using anti-HA antibody cross-linked to protein A-beads (second lane) or mock purified by protein A-beads (first lane). The proteins were eluted from the beads by incubating with HA peptide and then dialyzed as described under "Experimental Procedures." Aliquots of dialyzed extracts (25% of total) were separated by SDS-PAGE and visualized by silver staining. C, HA-Chk2 (10, 20, and 40 ng) purified from HCT116 cells without (lanes 1–3) or with (lanes 4–6) NCS treatment were incubated with 500 ng each of GST-p53 (1–82) and GST-Cdc25C (200–256). The reaction mixtures were separated by 8% SDS-PAGE, transferred to nitrocellulose, and analyzed by autoradiography (lanes 1–6, left panel) or by immunoblotting with either anti-GST or anti-HA antibodies (lanes 7–12, right panel). D, HA-Chk2 (5 ng) purified from HCT116 cells that were treated (lanes 3–6) or not (lanes 7–9) with γ irradiation (13 Gy) were incubated either with 500 ng of either GST-Cdc25C (200–256) (lanes 3, 5, and 8) or GST-p53 (1–82) (lanes 3, 4, and 7) and analyzed as in C. HA-Chk2 was mock-purified from HCT116 parental cells (as in B) with (lanes 10–12) or without (lanes 13–15) γ irradiation. The proteins were incubated with either GST-Cdc25C (lanes 11 and 14) or GST-p53 (lanes 10 and 13). GST-p53 (lane 1) or GST-Cdc25C (lane 2) were incubated with kinase buffer as controls. E, Chk2-FLAG (4, 8, 16, 32, or 100 ng) purified from recombinant baculovirus infected insect cells was incubated with 500 ng of GST-Cdc25C (200–256) (lanes 1–5) or 500 ng of GST-p53 (1–82) (lanes 6–10) in kinase buffer and analyzed as described in A. The proteins used were detected by immunoblotting with anti-GST and anti-FLAG antibodies (lanes 11–20). F, endogenous Chk2 was immunoprecipitated with anti-Chk2 antibody from HCT116 parental cells that were untreated (lane 1) or NCS-treated (500 ng/ml) for 2 h (lanes 2–4). Kinase activity of untreated Chk2 (2 μl of beads from untreated cells) was compared with that of NCS-activated Chk2 (2, 4, 6 μl beads) for phosphorylation of 500 ng of GST-p53 (1–82) and GST-Cdc25C (200–256). The amount of protein used in each assay was determined by immunoblotting as shown in the right panel. G, HA-Chk2 protein was purified from cells that had been treated with NCS for the indicated times (0–15 h). Chk2 protein (5 ng) from each time point was incubated with 500 ng of GST-p53 (WT) and GST-Cdc25C (200–256) in kinase buffer as described for C. The amount of proteins used in each assay was detected by immunoblotting with the indicated antibodies as shown in the right panel. Migration of phosphorylated GST-p53, HA-Chk2, and GST-Cdc25C are shown on the left.
RESULTS

Chk2 Is Not Activated to Phosphorylate p53 after DNA Damage—Chk2 isolated from cells that have sustained DNA strand breaks is greatly stimulated to phosphorylate Cdc25C at Ser216 when compared with Chk2 isolated from untreated cells (17–19). We were therefore interested in determining whether DNA damage to human cells would similarly activate Chk2 to phosphorylate p53. To characterize Chk2 protein kinase activity, we used clonally derived cells from the colorectal cancer cell line HCT116 that were engineered to stably express HA-tagged Chk2 (45). These cells express similar levels of stably expressed exogenous HA-Chk2 and endogenous Chk2 (Ref. 45 and Fig. 1A). HCT116 cells harbor wild type p53 that becomes stabilized after DNA damage (50). HA-Chk2 protein could be specifically immunopurified from HCT116 cells using anti-HA antibody cross-linked to protein A-Sepharose beads (Fig. 1B). We compared immunopurified HA-Chk2 from HCT116 cells that had been either not treated or treated with the radiomimetic agent NCS. As expected, the DNA-damaging treatment led to hyperphosphorylation of HA-Chk2 as evidenced by a mobility shift on SDS-PAGE, and HA-Chk2 from NCS treated cells was far more effective in phosphorylating a fragment of Cdc25C that contains Ser216, than HA-Chk2 from untreated cells (Fig. 1C). By contrast, a fragment of p53 that spans several of its p53 phosphorylation sites within the N terminus was phosphorylated less well by HA-Chk2 than Cdc25C, and, remarkably, there was virtually no increase in the ability of HA-Chk2 to phosphorylate p53 after NCS treatment (Fig. 1C).

A commonly used inducer of DNA strand breaks is γ irradiation, and so we performed a similar experiment comparing HA-Chk2 from γ irradiated or unirradiated HCT116 cells (Fig. 1D). The limitation in this case was that it was not possible to rapidly irradiate large quantities of these cells with ease, and thus smaller amounts of Chk2 were used. Nevertheless the results were essentially the same as with NCS. HA-Chk2 activity toward Cdc25C was significantly increased after γ irradiation, whereas that toward p53 was not detectably affected by irradiation. In this experiment we tested Cdc25C and p53 alone as well as together to demonstrate that the lack of increased phosphorylation of p53 after DNA damage is not due to competition with Cdc25C within the same reaction mixture for available kinase, a conclusion further supported by experiments shown below. Control purification from extracts of parental HCT116 cells revealed no contaminating kinases that were activated after γ irradiation to phosphorylate p53 or Cdc25C.

Previously we used recombinant baculovirus-expressed Chk2 tagged with the FLAG epitope at its C terminus (Chk2-FLAG) to phosphorylate p53. We wished to compare the relative ability of this source of enzyme to phosphorylate Cdc25C and p53 to ensure that the HA tag at the N terminus of Chk2 isolated from HCT116 cells was not affecting the ability of Chk2 to phosphorylate p53. Using similar constructs as substrates, we found that baculovirus-derived Chk2-FLAG is in fact dramatically more efficient at phosphorylating Cdc25C than p53 (Fig. 1E). Phosphorimaging analysis of the data shown in Fig. 1E indicated that Chk2-FLAG displayed up to a 50-fold greater ability to phosphorylate Cdc25C than p53 over the concentrations used. Importantly, Chk2-FLAG from baculovirus-infected insect cells strongly resembles HA-Chk2 from NCS-treated cells (51). First, like HA-Chk2 isolated from γ irradiated or NCS-treated cells, Chk2-FLAG is phosphorylated at Thr68 (the ATM kinase site that is required for Chk2 activation). Second, Chk2-FLAG has virtually identical specific activity toward Cdc25C as does HA-Chk2 isolated from NCS-treated HCT116 cells. Thus, two different sources of activated Chk2 are each dramatically more effective in phosphorylating Cdc25C than p53.

The possibility existed that endogenously expressed untagged Chk2 might behave differently than either stably expressed exogenous HA-Chk2 or baculovirus-derived Chk2-FLAG. To address this we examined the activity of Chk2 from the parental line of HCT116 cells using an anti-Chk2 monoclonal antibody. Consistent with our results using tagged forms of Chk2, endogenous Chk2 was far more active in phosphorylating Cdc25C than p53 and was stimulated by DNA damage to phosphorylate the former but not the latter (Fig. 1F). We considered the possibility that Chk2 may be activated to phosphorylate p53 at time points other than the single time point used thus far (i.e. 2 h after NCS treatment). HA-Chk2 was purified from HCT116 cells after exposure to NCS for various times and tested for its activity toward Cdc25C and full-length p53 at each time point. Increased activity of HA-Chk2 was detected as early as 30 min after treatment with NCS, and both then and at all time points thereafter, HA-Chk2 remained activated with respect to phosphorylation of Cdc25C but not of p53 (Fig. 1G). Similar results were obtained using a N-terminal fragment of p53 (data not shown).

In the kinase assays shown in Fig. 1, a GST-tagged N-terminal fragment of p53 was used as a substrate. Shieh et al. (39) had provided evidence that there are sites that are phosphorylated by Chk2 in other regions of the p53 protein. Furthermore, we previously demonstrated that p53 oligomerization is required for its efficient phosphorylation by Chk1 and Chk2 in vitro as well as its ability to be phosphorylated at N-terminal sites in vivo (39, 52). To determine whether the lack of tetramerization or distal phosphorylation sites in GST-p53 (1–82) explains the failure of phosphorylation by NCS-activated Chk2, tetrameric versions of p53 (full length or p53 lacking amino acids 1–96) were tested for their ability to serve as substrates for HA-Chk2 from untreated or NCS-treated cells (Fig. 2). Although NCS-activated HA-Chk2 was again far more effective in phosphorylating Cdc25C than HA-Chk2 from untreated cells, there was no discernable stimulation of its ability to phosphorylate two different tagged forms of full-length p53 or GST-p53. Note that bacterially expressed His-p53 forms

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Fig. 1—continued
tetramers (48), indicating that the ability of Chk2 to phosphorylate p53 does not result from a lack of oligomerization. This reinforces the conclusion that Chk2 cannot be activated to phosphorylate p53. Additionally, the result with His-tagged p53 shows that the GST tag is unlikely to interfere with Chk2 phosphorylation of p53. It should also be mentioned that these results are not unique to HCT116 cells; epitope-tagged Chk2 similarly isolated from a clonally derived HeLa cell line is also very weak in phosphorylating p53 and is not stimulated by NCS treatment. Thus, with or without activation by DNA damage, Chk2 from HCT116 cells displays only its basal phosphorylation activity toward p53.

Prephosphorylating p53 Does Not Make It a Better Substrate for Chk2—ATM can phosphorylate p53 at Ser15, and its ability to do so is increased when cells are irradiated or treated with NCS (7). Because ATM is upstream of Chk2, we considered the possibility that p53 needs to be first phosphorylated at Ser15 to be primed for phosphorylation at other N-terminal sites. A precedent for this is the observation that prephosphorylation of Ser15 is required for Thr18 phosphorylation by casein kinase 1 (31, 53). To test this we prephosphorylated p53 with the DNA-PK. This enzyme was used because it has not been possible to obtain a similarly purified and active preparation of ATM kinase in our laboratory. p53 was efficiently phosphorylated by DNA-PK at Ser15 as determined both by reactivity with an anti-Ser(P)15 antibody and an upward gel mobility shift after SDS-PAGE (Fig. 3A, top and bottom panels, compare lanes 1–4 with lane 5). Nevertheless, Chk2-FLAG showed no increase in its ability to phosphorylate p53 that had been prephosphorylated by DNA-PK (Fig. 3B, compare lanes 1–4 and lanes 5–8). This experiment argues against the possibility that p53 requires priming phosphorylation at Ser15 prior to Chk2 phosphorylation.

We then took advantage of our earlier observation that Chk1 can also phosphorylate p53 at a number of N-terminal sites to assay for any synergy between the two human Chk kinases (39). The possibility was considered that weak p53 phosphorylation by Chk2 could be a priming event for phosphorylation by Chk1 (or vice versa). In addition, it was of interest to see whether inter-Chk activating phosphorylation(s) can be demonstrated and are required for efficient phosphorylation of p53. Cdc25C and p53 were incubated with baculovirally expressed wild type or kinase-defective forms of tagged Chk1 and/or Chk2 proteins. Purity of proteins used are shown in the silver-stained gel in Fig. 4A. First, we found that, similar to Chk2, catalytic amounts of Chk1 are able to phosphorylate Cdc25C (200–256) but not the N terminus of p53 (Fig. 4B, lane 1). Second, extending observations with short peptides (54), Chk1 showed greater specific activity toward Cdc25C than did Chk2 (Fig. 4B, compare lanes 1 and 7). Third, incubation of Chk1 and Chk2 with p53 ruled out any requirement for both Chk kinases in phosphorylating p53 (Fig. 4B, lane 2). Finally, to explore the possibility of inter-Chk1 and Chk2 phosphorylation, we incubated wild type Chk1 with kinase-inactive Chk2 (11) or wild type Chk2 with kinase mutant Chk1 (10) (Fig. 4B, lanes 3 and 5) and found that neither Chk1 nor Chk2 can phosphorylate each other.

Taken together our data show that Chk1 and Chk2 behave very differently in in vitro kinase reactions toward two of their reported substrates p53 and Cdc25C. Although DNA damage greatly increases the activity of Chk2 toward Cdc25C, no similar augmentation of activity is observed on p53, as would be expected of checkpoint effector kinases functioning to trigger p53.

p53 Is Stabilized by DNA Damage in Cells Lacking Normal Physiological Levels of Chk1 and Chk2—Given our surprising observation that the p53 specific activity of Chk2 does not rise after checkpoint activation, a number of scenarios might be invoked to explain how Chk2 regulates p53. For example activation of an intermediary kinase, substrate recruitment by phosphorylation of an adaptor molecule, or other possibilities could explain our biochemical results. However, all of these ideas would predict that Chk2 controls p53 activation in vitro regardless of its performance in vivo. To test this hypothesis,
we used 21-nt siRNA duplexes to reduce Chk1 and Chk2 protein levels in cancer cell lines (49). Transfection of HCT116 cells with anti-Chk1, anti-Chk2, or both siRNAs but not the control anti-luciferase siRNA resulted in specific and significant down-regulation of each kinase. (Fig. 5, compare lanes 1–3 and 4–6 for Chk1 and lanes 7–9 for Chk2). We estimate that Chk2 was generally reduced by 60–75% and Chk1 by over 90–98% by their respective siRNAs. The relatively greater decrease in Chk1 may be because it is a less stable protein (55). Because Chk2 protein was incompletely ablated, we examined the impact of siRNA-induced Chk2 down-regulation on induction of Cdc25C phosphorylation at Ser216 using a phosphospecific antibody. NCS caused an increase in reactivity with the anti-Ser(P)216 antibody (Fig. 5, lanes 1–3) that was unaffected by nearly undetectable levels of Chk1 (lanes 4–6). However, reduction in Chk2 protein, although incomplete, was sufficient to inhibit increased phosphorylation of Ser216 on Cdc25C (Fig. 5, lanes 7–9). Transfection with the anti-luciferase or anti-Chk kinase siRNAs was not associated with changes in cell cycle distribution or apoptosis (data not shown). Because siRNA transfection was both effective and nonlethal, we went on to determine the impact of reduced Chk1 and Chk2 on p53 protein accumulation and transcriptional activation by p53. HCT116 cells transfected with the indicated siRNAs were treated with NCS for 0.5 or 3 h (Fig. 6, top left panel). p53 protein levels were increased after NCS treatment by 3 h, and transfection of the luciferase control siRNA did not prevent p53 induction. However, down-regulation of Chk1, Chk2, or even both kinases simultaneously had no effect on p53 stabilization. We next examined longer time points, 5 and 15 h, following NCS treatment before assessing p53 levels (Fig. 6B, right panels). Again, very low levels of Chk2 and virtual elimination of Chk1, either
individually or both proteins together, had no effect on induc-
tion of p53 after NCS treatment. (Note that the anti-Chk2
polyclonal antibody used in these studies has a reduced ability
to recognize Chk2 after NCS treatment presumably because
the kinase becomes highly phosphorylated as a result of NCS
treatment. This phenomenon has been noted with other Chk2
antisera (11).)

Interestingly, down-regulation of Chk1 and Chk2 without
drug treatment stabilized p53 to some extent, suggesting that
cells may sense a loss of Chk1 or Chk2 as a stress signal.
However, this stress did not compromise p53 stabilization fol-
lowing NCS treatment because peak levels achieved in control
versus Chk1 and/or Chk2 siRNA transfected cells were compa-
rible or were even slightly increased by down-regulation of the
Chk kinases (Fig. 6A, left and right histograms).

p53 mediates its checkpoint function primarily through tran-
scriptional activation. It was of interest to determine whether
p53 can induce its transcriptional targets when the checkpoint
kinases were reduced or depleted by siRNA (Fig. 6). Three well
validated targets of p53 (p21, Mdm2, and p53-induced gene 3) were examined by immunoblotting in parallel with p53, Chk1,
and Chk2. Within 30 min after NCS treatment and prior to p53
stabilization, there was a consistent slight decrease in the
levels of detectable p21 and Mdm2 protein regardless of the
siRNA that was introduced into the cells. Although the reason
for this is not clear, by 3 h after the addition of NCS concomi-
tant with increased p53, a modest increase in accumulation of
p21 was observed (Fig. 6A, left p21 panel) that became more
pronounced with longer intervals after drug treatment (Fig.
6B, right p21 panel). Hdm2 and p53-induced gene 3 protein
levels were also increased upon stabilization of p53 regardless
of the levels of Chk1 and Chk2. Note as well that Jallepalli
et al. (61) have determined that p53 induction as well as cell cycle
arrest and apoptosis after DNA damage are normal in HCT116
cells from which both Chk2 alleles have been deleted.

Because Chk2 is thought to be responsible for phosphoryla-
tion of p53 on Ser20, we sought to directly examine the effect
of Chk2 down-regulation on induction of phosphorylation at that
site. To be able to assess Ser20 phosphorylation independently
of changes in p53 protein levels, we took advantage of an
earlier observation that mutant p53 in HT-29 human colorectal
carcinoma cells is phosphorylated but not stabilized after DNA
damage (52). HT-29 cells were transfected with luciferase or
Chk2 siRNA as above and then collected 30 min after exposure

Fig. 6. Down-regulation of Chk1 or Chk2 do not affect p53 levels in cells with or without DNA damage. HCT116 transfected with the
indicated siRNAs were either not treated (−) or treated with 500 ng/ml NCS for either 0.5 and 3 h (A) or 5 and 15 h (B) as indicated. The extracts
were resolved by SDS-PAGE and subjected to immunoblotting with anti-Chk1, -Chk2, -p53, actin, p21, HDM2, p53-induced gene 3 or Cdc25C
Ser(P)216 antibodies as indicated. The bottom panels show quantitation of fold p53 induction relative to lane 1 (control transfected untreated cells)
and normalized to actin using Image J software (National Institutes of Health). C, HT-29 cells with mutant p53 were transfected with siRNA
targeting luciferase (Luc) or Chk2 and either untreated (−) or exposed to 10 Gy IR (+) and collected after 30 min. The cell extracts were resolved
by SDS-PAGE and immunoblotted with anti-Chk2, p53, anti-phospho-p53 Ser(P)20 or actin antibodies as indicated on the right. IR, irradiation.
that cells were exposed to 500 ng/ml NCS for 5 h. Immunoblotting with antibodies against Chk1, Chk2, p53, actin, or Hdm2 as indicated on right. siRNAs and treated or not (H11002) with 500 ng/ml NCS for either 0.5 or 2.5 h. The extracts were resolved by SDS-PAGE and subjected to luciferase. The overlap between checkpoint pathways in yeast and mammalian cells is striking and suggests that DNA damage surveillance mechanisms have evolved to produce a common set of cellular responses. Findings supporting that p53, a well authenticated checkpoint factor, is a likely target of Chk2 were therefore both expected and consistent with many other aspects of the mammalian DNA damage response pathway. Nevertheless, the data presented in this study (and in Ref. 61) must be taken as a serious challenge to the notion that in all cases Chk2 lies upstream of p53 in the response to γ irradiation or radiomimetic drugs.

How do we reconcile our results with those previously published showing that recombinant Chk2 phosphorylates p53 and abrogation of Chk2 function disables the p53 response? With respect to the first, one must consider that substrate specificity of activated kinases is rendered not only by identity of the residues being phosphorylated but also optimal interaction between surrounding residues in the substrate and in the active site of the kinase. Using peptide libraries to search for substrate motifs for Chk1 or Chk2 phosphorylation, it was found that both kinases display strong preference for the sequences surrounding Cdc25C and Cdc25A sites and virtually none for the Ser20 region of p53 (54, 56). This is consistent with the fact that both kinases display strong preference for the sequences surrounding Cdc25C and other reported Chk2 targets including BRCA1 (54). Although short peptides might not have provided the same information as a larger protein, our data clearly agree with their findings. In our experiments, Chk2 favored Cdc25C over p53 by a factor of 50-fold. Our previous experiments (39) and those of Chehab et al. (30) showing phosphorylation of p53 by these kinases used stoichiometric amounts of substrates and kinases, an experimental condition where substrate specificity of kinases cannot be properly measured. Although Chk2 can interact with p53 (57),3 this association is not sufficient to allow Chk2 to phosphorylate p53 comparably with Cdc25C.

To examine the significance of our biological observations in vivo, we used siRNA to down-regulate Chk1 and Chk2 in tumor cell lines. Although Chk1 was very efficiently ablated, in our

3 J. Ahn and C. Prives, unpublished data.
experiments Chk2 could only be reduced by 60–70%. Nevertheless, this amount of down-regulation was sufficient to prevent DNA damage-induced phosphorylation of Cdc25C at Ser\textsuperscript{205} but did not effect p53 activation and function. Admittedly, this is an imperfect control given the likelihood that distinct regulatory mechanisms exist for the various substrates of a given kinase. Arguing against such a criticism in this case is that in vitro Cdc25C is a much stronger substrate than p53, suggesting that Cdc25C should have a higher ablation effect threshold in vivo than p53. We do not observe such a correlation. Nevertheless in RNAi experiments, aphenotypic observations in the context of incomplete knock-down leave open the possibility that the remaining protein is sufficient to mediate function. These concerns are for the most part alleviated by the data presented by Jallepalli et al. (61) that homozygous deletion of Chk2 does not have an impact on p53 function. Moreover, introduction of Chk1 siRNA into Chk2\textsuperscript{2/−} cells revealed no diminution of p53 accumulation or function.

Previous studies examining Chk2 and p53 showed that introduction of a dominant negative form of this kinase (30) led to failure of U2OS cells to stabilize or phosphorylate endogenous p53 at Ser\textsuperscript{205}. It can be argued that introduction of a dominant negative form of Chk2 might have effects in cells in addition to Chk2 expression. In fact, reports indicate that some tumor-derived mutants of Chk2 do not act in a dominant negative fashion when expressed with wild type Chk2 (45, 46). In our previous study (39) we showed that co-transfection of antisense Chk1 with p53 into H1299 cells led to reduced levels of p53 when compared with its being co-transfected with vector alone. Although this is not consistent with our results herein, it is possible that the overexpression of transfected p53 requires factors mediating p53 activation is now in place, and such as Thr\textsuperscript{18} or Ser\textsuperscript{20}. An outline for future identification of those factors mediating p53 activation is now in place, and exciting new findings in these areas are anticipated.

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