Modulation of p53 Binding to Holliday Junctions and 3-Cytosine Bulges by Phosphorylation Events†

Deepa Subramanian and Jack D. Griffith*

Lineberger Comprehensive Cancer Center and Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295

Received June 23, 2004; Revised Manuscript Received December 6, 2004

ABSTRACT: Recognition of certain types of DNA lesions by the tumor suppressor protein, p53, represents one of the several downstream functions of this protein in response to DNA damage. This binding property is regulated by several factors including posttranslational modifications and interactions with other proteins. Phosphorylation by several stress-response kinases activates p53 by increasing protein stability as well as transactivation properties. Here we examined the effect of phosphorylation events on the sequence-independent binding properties of p53 using two DNA substrates: One resembling Holliday junctions and the other containing extra base bulges. Gel retardation assays showed that dephosphorylation of serine 392 in the C-terminal domain of p53 greatly reduces Holliday junction and lesion recognition. In contrast, sequence-specific binding is disrupted by the removal of some N-terminal phosphates but not serine 392. Rephosphorylation of p53 by certain kinases can restore p53 recognition of Holliday junctions and 3-cytosine bulges. In all cases, phosphorylation of serine 392 occurs; however, reactivation also involves other residues. Together, the results show that p53 DNA binding activity is strongly regulated by the phosphorylation state of the protein.

The tumor suppressor protein, p53, plays an important role in the maintenance of genomic integrity following DNA damage. The best understood function of p53 is its ability to prevent growth of abnormal cells. Levels of p53 rise immediately after genomic stress due to lengthening of the protein half-life. Increased p53 stability occurs through modifications at the protein level (*1*−*3*) as there is no elevation in p53 mRNA after genomic insult (*4*). Modifications to the protein may also directly activate p53 cellular functions. Mechanisms that regulate p53 stability and function include posttranslational modifications such as phosphorylation, acetylation, and sumoylation, interactions with other proteins, and control of protein subcellular localization as well as conformation changes in the protein (reviewed in refs *5* and *6*). All of these factors maintain tight control of p53 in normal cells but allow rapid protein activation in response to DNA damaging agents.

One of the most studied functions of p53 is transactivation of downstream genes that are required for cell cycle arrest and apoptosis following DNA damage (reviewed in refs *7* and *8*). As a result, cells either undergo DNA repair prior to cell cycle progression or proceed to cell death. This activity requires efficient binding of the protein to sequence-specific response elements in the promoter regions of target genes. There is growing evidence that a second role for p53 is sequence-independent or structure-specific binding which acts as a signal for downstream repair and recombination pathways. p53 binds with high affinity to single strand and double strand ends (*9*), which are products of ionizing radiation, and extra base bulges (*10*), which result from polymerase errors and Holliday junction structures (*11*, *12*), as well as three-stranded intermediates (*11*, *13*) that form during homologous recombination and nonhomologous sister chromatid exchange following UV irradiation. We have shown that p53 can also bind to some base−base mismatches (*14*). Recognition and resolution of these structures are required before the cell can progress to mitosis.

Increased phosphorylation at several residues following DNA damage has been demonstrated using antibodies that recognize specific modified amino acids. For example, p53 from cells exposed to UV or ionizing radiation shows increased phosphorylation at serines 6, 9, 15, 20, 33, 37, and 46 as well as threonines 18 and 81 (ref *15* and references cited therein). Elevated phosphorylation of serine 15 also occurs following treatment with agents such as cisplatin (*16*) and camptothecin (*17*), as well as human fibroblasts undergoing replicative senescense or ras-induced premature senescence (*18*, *19*), making it a key residue in the regulation of p53 response. Phosphorylation of these N-terminal residues appears to regulate protein stability. Several C-terminal residues are also modified following UV and ionizing radiation. Both serines 315 and 392 are modified following UV radiation while serine 315 alone is phosphorylated following ionizing radiation (*20*, *21*). It has been suggested that modified residues in the C-terminus tend to participate in activation of p53 function (*6*).

Stress-response kinases that phosphorylate the N-terminus of p53 in vivo include ATM (*22*−*24*), ATR (*23*, *25*), Chk1 (*26*), and Chk2 (*26*) which increase protein stability. Additionally, ATM and ATR can also enhance transcriptional

† This work was supported in part by the Ellison Medical Foundation and by National Institutes of Health Grants CA19014 and GM31819.

* To whom correspondence should be addressed. Tel: 919-966-2151. Fax: 919-966-3015. E-mail: jdg@med.unc.edu.

10.1021/bi048700u CCC: $30.25
Published on Web 01/28/2005

activation by directly phosphorylating serines 15 and 37 (*27*, *28*). In vitro, numerous kinases have been shown to phosphorylate p53 such as DNA-dependent protein kinase (DNA-PK)[1] at serines 15 and 37 (*29*), casein kinase I (CKI) at serines 6 and 9 (*30*), mitogen-activated protein kinase at threonines 73 and 83 (*31*), and several cyclin-dependent kinases (*32*−*34*). In vitro phosphorylation of some C-terminal serines by protein kinase C (PKC) and casein kinase II (CKII) has been shown to enhance p53 DNA binding as well as transcriptional activation (*35*, *36*). Finally, enhancement of p53 DNA binding and transcription activity can also occur as a result of acetylation of lysine 382 by p300/CBP (*27*) and sumoylation of several lysine residues (*37*−*39*).

It is clear that, following genotoxic stress, posttranslational modifications occur which increase both p53 stability and activity. The increased stability and accumulation of p53 in the nucleus were believed to occur due to disruptions of p53−MDM2 interactions as a result of modifications to the MDM2 binding domain (*40*). However, recent evidence has shown that increased stability occurs more due to higher CBP/p300 binding rather than a reduction in MDM2 binding (*28*). Additionally, stabilization of p53 may occur due to modifications of other p53 interacting proteins. For example, treatment of cells by several DNA damaging agents may result in the inhibition or relocalization of MDM2, resulting in increased levels of p53 (*15*).

The effect of these posttranslational modifications on p53 DNA binding activity is poorly understood. It is not clear if increased binding to response elements in the promoter regions of target genes is simply due to elevated protein levels or if phosphorylation can directly activate the DNA binding response. For example, addition of negative charges by phosphorylation could induce conformation changes that prevent the C-terminus/core interactions. The last 30 amino acids of p53 have been shown to negatively regulate its sequence-specific binding activity (*21*, *40*), and posttranslational modifications of this region may relieve the inhibition, thus resulting in increased p53 DNA binding activity. Modifications of C-terminal residues may also directly impact on p53 lesion recognition properties. The C-terminus is responsible for sequence-independent DNA binding, and data from our laboratory have shown that this domain is sufficient for the recognition of insertion/deletion lesions (*10*). Thus, modifications of the C-terminus should significantly influence p53 recognition of DNA structures. Other postdamage pathways may also require activated p53 for their function. For example, recent reports have shown that global genomic nucleotide excision repair requires p53 activity while transcription-coupled repair does not (*41*, *42*).

To determine the effect of phosphorylation on p53 structure-specific binding properties, protein phosphatases and kinases were utilized to modify p53 in vitro. The activity of the altered proteins was monitored by mobility shift assays using two probes: one containing 3-cytosine bulges and the other resembling Holliday junctions. Binding activity was compared to the effects of posttranslational modifications on binding to its consensus sequence. Our results show that dephosphorylation by protein phosphatase 1 (PP1) reduces p53 binding to Holliday junctions and 3-cystosine bulges as well as the consensus sequence. Dephosphorylation by λ-phosphatase (λP) does not have a significant effect on Holliday junction and 3-cytosine bulge binding but still reduces consensus sequence recognition. One difference between these two enzymes is that λP does not dephosphorylate the C-terminal serine 392 while PP1 does, suggesting that this residue may play an important role in Holliday junction and lesion recognition. Phosphate groups were added back to dephosphorylated p53 using various kinases, and some were effective in restoring binding to Holliday junctions and 3-cytosine bulges. However, since multiple sites were phosphorylated by each kinase, the exact residues involved in sequence-independent binding remain unclear. Overall, the data show that phosphorylation of p53 plays a crucial role in detection and interaction with sites of DNA damage and unusual DNA structures.

EXPERIMENTAL PROCEDURES

Proteins. Human p53 was overexpressed in insect SF9 cells using a baculovirus vector provided by Dr. Arnold Levine and purified as described previously (*43*). Protein was purified to 95% homogeneity, and concentrations were determined by Bradford assays. PP1, λP, PI-2, CKI, CKII, and cdc2 protein kinase (cdc2) were obtained from New England Biolabs (Beverly, MA). PKC and DNA-PK were obtained from Promega, Inc. (Madison, WI). Chk1 and Chk2 kinases were obtained from Upstate Ltd. (Lake Placid, NY).

DNA Probes. Oligonucleotides were obtained from MWG Biotech (Greensboro, NC). Holliday junction, 3-cytosine bulge, and duplex probes were synthesized as described previously (*12*). The consensus sequence probe was prepared by annealing a 49-nt oligonucleotide (5′-GCTCGAACCC-**TACAGAACATGTCTAAGCATGCTGGGG**ATCCGC-CGCAC-3′) to its complementary sequence (5′-GTGCG-GCGGAT**CCCCAGCATGCTTAGACATGTTCTGTA**-GGGTTCGAGCC-3′), with the response element shown in bold. To make probes for mobility shift assays, one strand of each probe was first end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA), followed by the addition of equal amounts of the other strands. The samples were heated to 65 °C for 10 min in the presence of 0.4 M NaCl and allowed to cool slowly to room temperature overnight, and annealed products were purified on 10% nondenaturing polyacrylamide gels.

Preparation of Nuclear Extracts. 293 human embryonal kidney cells (75% confluent in 150 mm Petri plates) were washed twice with cold 1× PBS. Lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM PMSF) was added at 2 mL per plate, and cells were incubated at room temperature for 5 min. The cells were then scraped off the plates, transferred to microcentrifuge tubes, and sonicated for 10 s, and lysates were clarified by centrifugation at 13000*g* for 10 min. Protein concentrations were determined using Bradford assays.

In Vitro Dephosphorylation. Purified p53 (1.8 pmol of tetramers) was treated with either 5 units of PP1 or 800 units

[1] Abbreviations: PP1, protein phosphatase 1; λP, λ-phosphatase; CKI, casein kinase I; CKII, casein kinase II; DNA-PK, DNA-dependent protein kinase; PKC, protein kinase C; cdc2, cdc2 protein kinase; PI-2, protein phosphatase inhibitor 2.

of λP (one unit is defined as the amount of enzyme that hydrolyzes 1 nmol of *p*-nitrophenyl phosphate in 1 min) in 20 μL reactions containing the p53 binding buffer (10 mM Hepes−KOH, pH 7.8, 25 mM KCl, 0.5 mM DTT, 10% glycerol, 2 mM $MnCl_2$, and 25 ng of salmon sperm DNA as nonspecific competitor) at room temperature for 20 min. For immunoblot analysis, an equal volume of 25 mM sodium phosphate, pH 6.5, was added, and the reactions were applied to PVDF membranes (Immobilon-P; Millipore, Billerica, MA) using a dot blot manifold and processed as described below.

In Vitro Kinase Assays. Prior to kinase assays, purified p53 was first dephosphorylated using PP1 (5 units of PP1/1.8 pmol of p53) and the phosphatase inhibited by the addition of PI-2 (1 unit/5 units of PP1), a type I protein phosphatase inhibitor. To examine total phosphorylation of p53, 1.8 pmol of the protein (tetramer) was treated with 200 units (where 1 unit can transfer 1 pmol of phosphate groups per minute) of CKI, CKII, cdc2, Chk1, Chk2, PKC, and DNA-PK in 20 μL reactions containing the p53 binding buffer (replacing $MnCl_2$ with 50 mM $MgCl_2$) and 0.1 μCi of [γ-^{32}P]ATP for 20 min at room temperature. Products were analyzed by 10% SDS−PAGE and autoradiography. PKC reactions were supplemented with phosphatidylserine (0.6 μg/μL) to activate the enzyme.

For detection of site-specific phosphorylation by immunodot blotting, kinase assays were set up as described above except that [γ-^{32}P]ATP was replaced with 25 μM unlabeled ATP. Following incubation, an equal volume of 25 mM sodium phosphate, pH 6.5, was added, and samples were immobilized on PVDF membranes using a dot blot vacuum manifold. The membranes were subjected to immunoblot analysis as described below.

Mobility Shift Assays. End-labeled probes (0.18 pmol) were added to 20 μL reactions containing p53 (1.8 pmol) that was treated with phosphatases or kinases as described above and incubated for 15 min at room temperature. Control reactions contained untreated p53. The reactions were adjusted to 10% glycerol, 0.025% bromophenol blue, and 0.025% xylene cyanol and products separated on 5% nondenaturing polyacrylamide gels in 0.5× TBE (45 mM Tris−borate, 1 mM EDTA). The gels were run at 200 V for 2.5 h at 4 °C, dried, analyzed by autoradiography, and quantified using a Storm 840 phosphoimager (Molecular Dynamics, Piscataway, NJ). ImageQuant (Molecular Dynamics) software was used to determine the amount of free probe and protein-bound probe and the total amount of input probe in each reaction. Complex formation was calculated as a percentage of total input DNA.

Immunoblot Analysis. Polyclonal antibodies to specific p53 phosphoserine residues were obtained from Cell Signaling, Inc. (Beverly, MA). PVDF membranes with immobilized proteins were completely dehydrated with methanol (following the manufacturer's instructions), incubated in a 1:1000 dilution of the phosphoserine-specific polyclonal antibodies in 1× TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% instant nonfat dried milk overnight at 4 °C, washed three times for 10 min each in TBST, incubated in a 1:5000 dilution of goat anti-rabbit conjugated to horseradish peroxidase in 1× TBST for 1 h at room temperature, and washed three times for 10 min each in TBST, and blots were detected by chemiluminescence (ECL kit; Amersham, Piscataway, NJ). Immunoblots were quantified by densitometry.

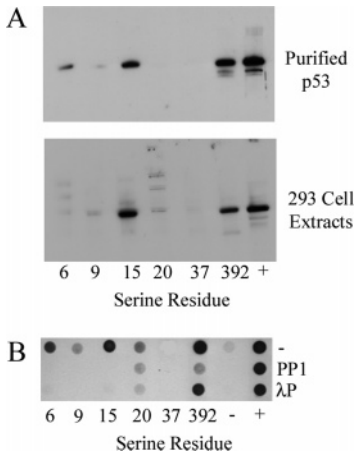

FIGURE 1: (A) Analysis of p53 phosphorylation sites. p53 purified from baculovirus-expressing insect cells (60 ng per lane) or whole cell extracts from 293 cells (10 μg per lane) were subjected to western blot analysis using rabbit polyclonal antibodies against specific phosphoserine residues. A polyclonal antibody to total p53 was used as a positive control (+). (B) Dephosphorylation of p53. Purified p53 (90 nM) was treated with no enzyme (−), 5 units of PP1, or 800 units of λP for 20 min at room temperature. Samples were applied to nitrocellulose membranes using a dot blot manifold followed by immunoblotting using the phosphoserine-specific antibodies, no primary antibody (−), or a polyclonal antibody to total p53 (+).

RESULTS

The Phosphorylation State of Purified p53 Resembles the in Vivo Situation. Studies from our laboratory have shown that p53 purified from baculovirus-infected insect cells can effectively bind Holliday junctions, 3-cytosine bulges, and some base/base mismatches as well as its consensus sequence (*10*, *11*, *14*). Since posttranslational modifications play an important role in p53 DNA binding properties, the phosphorylation state of the protein purified from baculovirus-infected insect cells was examined by western blotting using phosphoserine-specific antibodies to the following p53 residues: serines 6, 9, 15, 20, 37, and 392. Figure 1A shows that purified p53 was phosphorylated at serines 15 and 392, to a lesser extent at serine 6, and very weakly at serine 9. However, there may be other phosphate-containing residues that we were unable to detect due to lack of appropriate reagents. To determine if the phosphorylation state of baculovirus-expressed p53 reflects that of cellular p53, lysates from 293 cells were used as controls in the western blot analysis. The results (Figure 1A) show that, in 293 cells, p53 phosphorylation was observed at serines 15 and 392 and very weakly at serine 9. Antibodies to serines 15 and 392 are quite specific as only very low levels of cross-reactivity to other nuclear proteins were detected. Antibodies to phosphoserines 6 and 20 showed multiple bands in the western blots, indicating cross-reaction with other proteins in the sample; however, both residues in p53 appear to be phosphorylated to a slight extent. No phosphorylation was seen at serine 37. Additionally, p53 phosphorylation patterns in primary foreskin fibroblast cells matched that seen in 293 cells (data not shown). These results correlate with previous reports that p53 purified either from baculovirus-infected cells (*44*) or from undamaged human cells (*45*) contains

posttranslational modifications. However, the fraction of total protein that is phosphorylated cannot be determined using these assays. Overall, the results indicate that p53 expressed in insect cells is predominantly phosphorylated at serines 15 and 392 and reflects the state of the native cellular protein, thus supporting the use of baculovirus-infected cells as a valid source of functional protein in DNA binding studies.

PP1 Can Dephosphorylate both N- and C-Terminal Serine Residues. To remove preexisting phosphate groups from purified p53, the protein was treated with PP1 and λP, and the loss of phosphate groups from serine residues was monitored by immunodot blot analysis using the phosphoserine antibodies. Since the western blots showed these reagents to be very specific to the purified protein, we were confident that signals from the dot blot analysis accurately reflected p53 phosphorylation states. Figure 1B shows that both phosphatases effectively removed the phosphate groups from serines 6 and 15 after 20 min of treatment. Low levels of phosphorylation were also seen at serines 9 and 20, which were removed by both phosphatases in the case of serine 9. However, complete dephosphorylation of serine 20 was not seen; approximately 20% and 10% of the signal remained after treatment with PP1 and λP, respectively. Serine 392, on the other hand, was dephosphorylated by PP1 but not λP. Dephosphorylation of serine 392 by λP was not detected even at higher concentrations of the enzyme (data not shown). The dephosphorylated products were also probed with an antibody to total p53 to ensure that equal amounts of protein were present in each reaction.

Dephosphorylation of Serine 392 Inhibits p53 Binding to DNA Lesions. Dephosphorylated p53 was tested for structure-specific binding by mobility shift assays using two different probes: templates resembling Holliday junctions or containing a cluster of three 3-cystosine bulges to represent insertion/deletion mismatches. Both of these probes have been used extensively by our laboratory in binding studies with p53 and mismatch repair proteins (*10*−*12*). As a control, a consensus sequence probe was used which contains the 27 bp human GADD45 p53 response element at the center (*14*). The probes were incubated with p53 at a molar ratio of 1:10 (DNA:protein tetramers) for 20 min at room temperature, and protein/DNA complexes were separated from free DNA by nondenaturing polyacrylamide gel electrophoresis. As seen in Figures 2A and 3A, p53 bound with high affinity to Holliday junction probes and 3-cytosine bulges. p53 purified from insect cells was also able to bind the consensus sequence probe (Figure 3B). Binding to these probes is specific as no p53 complexes were formed with a duplex probe lacking lesions or the consensus sequence (data not shown). Since phosphatases require the presence of Mn^{2+} for efficient activity and this is not present in our standard p53 binding buffer, mobility shift assays were performed to ensure that this metal ion does not interfere with p53/DNA complexes. The results show that the presence of Mn^{2+} in the reactions did not affect p53 binding to lesions or the consensus sequence (Figures 2 and 3).

When p53 dephosphorylated with PP1 was incubated with the Holliday junction probe, a loss of signal at the position of the protein/DNA complexes was seen (Figure 2A, lane 4). However, additional supershifted complexes were also seen which probably represent aggregates of PP1 with p53 and the probe. Treatment with λP also resulted in a slight

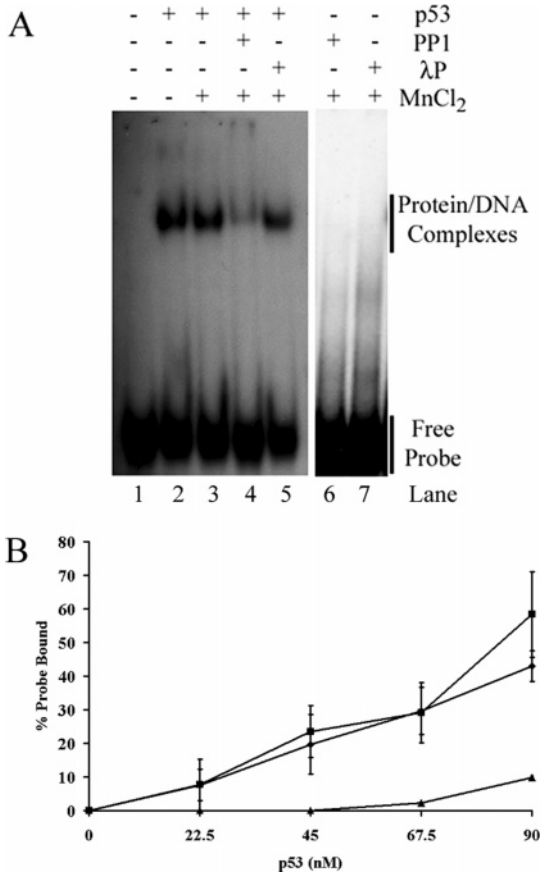

FIGURE 2: Effect of dephosphorylation on p53 binding to Holliday junctions. (A) p53 (90 nM) was treated with 5 units of PP1 (lane 4) or 800 units of λP (lane 5) or left untreated (lanes 2 and 3) for 20 min at room temperature. Labeled Holliday junction probes (9 nM) were added to each reaction for an additional 15 min. Reaction products were separated on a 5% nondenaturing polyacrylamide gel followed by autoradiography. In lanes 6 and 7, probes were incubated with the phosphatases alone. Brackets indicate the position of the protein/DNA complexes and free probe. (B) Binding isotherms for the different forms of p53 were generated by incubating Holliday junction probes with increasing concentrations of native p53 (■), p53 treated with PP1 at 0.03 unit/nM protein (◆), or 0.06 unit/nM protein (▲) followed by mobility shift assays. Protein/DNA complexes were quantified as described in Experimental Procedures and represented as percent probe bound and plotted against p53 concentration. Error bars represent values from two experiments.

reduction in p53/Holliday junction complexes (Figure 2, lane 5) but not to the extent seen with PP1. Neither phosphatase bound directly to the Holliday junction probe (Figure 2A, lanes 6 and 7), indicating that loss of complexes was not due to competition for the probe but a direct result of p53 dephosphorylation. Since activity of the dephosphorylated protein can be restored by treatment with certain kinases (see below), it is not likely that the inhibition of DNA binding activity was due to contaminants that inactivate p53. Quantification of PP1-treated protein/DNA complexes showed that 67% of the input DNA was free probe, 21% was bound as p53/DNA complexes, and 12% formed aggregates that are retained in the well (Table 1). Overall, 33% of the DNA is bound in complexes compared to 52% with untreated p53, which represents a 36% reduction in DNA binding. Treatment with λP slightly reduced p53/Holliday junction complexes.

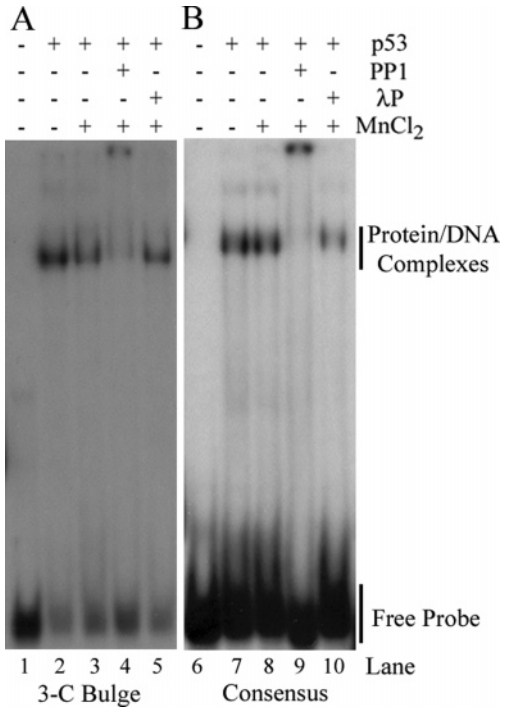

FIGURE 3: Binding of dephosphorylated p53 to the 3-cytosine bulge and consensus sequence probes. p53 was dephosphorylated as described in Figure 2 followed by further incubation with end-labeled probes containing either a 3-cytosine bulge (A) or the p53 consensus sequence from the Gadd45 promoter (B). Reaction products were separated on 5% nondenaturing polyacrylamide gels and visualized by autoradiography. Brackets indicate the position of the free probe and protein/DNA complexes.

To assess the relative affinities of dephosphorylated p53 versus unmodified protein, PP1 treatment was carried out at two different concentrations, and binding isotherms were generated with mobility shift assays (Figure 2B). K_D values were determined as the concentration at which 50% of the DNA is bound by protein. Untreated protein had a K_D of 4.9 nM, while p53 treated with a lower concentration of PP1 was 5.8 nM, and this rose to 24.6 nM at the higher concentration, indicating a 5-fold decrease in DNA binding activity.

When p53 dephosphorylated with PP1 was incubated with the 3-cytosine bulge probe, a loss of signal at the position of the protein/DNA complexes similar to the Holliday junction probe (Figure 3A, lane 4) was observed. Quantification of the sample showed that ~71% of the input DNA was free probe, ~16% was complexed with p53, and 14% was aggregated in the well (Table 1). Thus, the amount of probe that was bound in this sample was 30% compared to 46% complex formation with untreated p53 (35% decrease in complex formation). Treatment with λP did not alter p53 binding to the 3-cytosine bulge probe.

A loss of signal at the position of p53/DNA complexes along with the formation of aggregates in the well was also seen with PP1-treated p53 and the consensus sequence probe (Figure 3B, lane 9). Quantification of the sample showed that ~77% of the input DNA was free probe, 13% was in the p53/DNA complexes, and 10% was aggregated in the well. This represents a total of 23% probe bound compared to 41% with untreated p53 (44% decrease in complex formation). A significant decrease in complex formation was also seen with p53 treated with λP. No aggregates were seen in the well in this sample, and complex formation was reduced by 51%. Since treatment with PP1 dephosphorylates serine 392 and also shows greater inhibition of lesion binding, we can conclude that this residue may play an important role in DNA damage recognition. However, other sites could also be involved which were not tested for in this study.

CKI, CKII, Chk1, DNA-PK, and PKC Can Phosphorylate p53 in Vitro. p53 dephosphorylated with PP1 was rephosphorylated using the following kinases: CKI, CKII, cdc2, Chk1, Chk2, DNA-PK, and PKC. Prior to kinase treatment, the phosphatase was stopped by the addition of PI-2, a type 1 protein phosphatase specific inhibitor. Protein phosphorylation was monitored in two ways. First, overall phosphorylation of p53 was monitored by treating the protein with various kinases in the presence of [γ-^{32}P]ATP. Products were analyzed by SDS−PAGE and autoradiography. Figure 4A shows that PKC and CKII were most efficient in phosphorylating p53, followed by DNA-PK and Chk1 and finally CK1. No phosphorylation was detected with cdc2 and Chk2. Second, site-specific phosphorylation was determined by repeating the kinase assays with unlabeled ATP and analyzing the reaction products by immunoslot blot analysis using specific phosphoserine antibodies (Figure 4B). Since cdc2 and Chk2 showed no labeling with [γ-^{32}P]ATP, these were not used for further experiments. Untreated p53 showed the expected phosphorylation pattern at serines 6, 15, and 392. p53 treated with PP1 showed no signal at any of the residues tested. All of the kinases tested restored serine 392 phosphorylation to the levels of the native protein. Quantification of the serine 392 signal showed no significant difference between untreated and kinase-treated p53. Phosphorylation at two novel sites, serines 20 and 37, was also seen with CKI as well as a weaker signal at serine 9. Treatment with CKII restored serines 6 and 15 although levels at serine 6 are much higher than those of the native protein while serine 15 was at the same level as the original source. Chk1 strongly phosphorylates serines 6, 15, and 37 with levels being much higher than the native protein. Weak phosphorylation of serine 20 was also seen. DNA-PK similarly showed higher levels of phosphorylation at serines 15 and 37. PKC was the only kinase that did not have a strong effect on the residues that were tested even though high levels of phosphorylation were seen in the presence of [γ-^{32}P]ATP, indicating modification at sites other than the ones tested in this study.

Rephosphorylation of Serine 392 Restores p53 Structure-Specific Binding Activity. Phosphorylated p53 was tested by mobility shift assays for binding to both Holliday junctions and 3-cytosine bulges. Dephosphorylated p53 (as described above) was first treated with the various kinases for 20 min followed by the addition of the probes for an additional 15 min. None of the kinases tested bound directly to the Holliday junction substrate (Figure 5, lanes 1−7) including DNA-PK which has intrinsic DNA binding activity. This is probably due to the reaction conditions used in the gel shift assays (see Discussion). As expected, untreated p53 bound with high affinity (~46%) to Holliday junctions while dephosphorylated p53 bound poorly (19%) (Figure 5, lanes 13 and 14, and Table 2). Addition of phosphate moieties by CKI, Chk1, and PKC restored p53 binding to Holliday junctions to the same level as the native protein. On the other hand, treatment with CKII had no effect as complex formation was at the

Table 1[a]

	Holliday junctions			3-cytosine bulge			GADD45 promoter		
		bound probe			bound probe			bound probe	
	free probe	p53 complex	aggregate	free probe	p53 complex	aggregate	free probe	p53 complex	aggregate
no protein	100	0	0	100	0	0	100	0	0
p53	47.9 ± 13.2	52.1 ± 13.2	0	53.8 ± 15.0	46.2 ± 15.0	0	58.9 ± 20.6	41.1 ± 20.6	0
p53 + PP1	67.2 ± 18.3	21.0 ± 12.5	11.9 ± 6.2	70.6 ± 17.8	15.8 ± 13.3	13.6 ± 7.2	76.9 ± 15.7	13.3 ± 12.6	9.9 ± 7.4
p53 + λP	54.1 ± 16.3	45.4 ± 15.6	0	52.1 ± 15.6	48.0 ± 15.6	0	80.0 ± 11.3	20.0 ± 11.3	0

[a] p53/DNA complexes and unbound probe from mobility shift assays were quantified as described in Experimental Procedures and represented as a percentage of total input DNA (free probe in the no protein sample was set as 100%). Values represent data from four independent experiments.

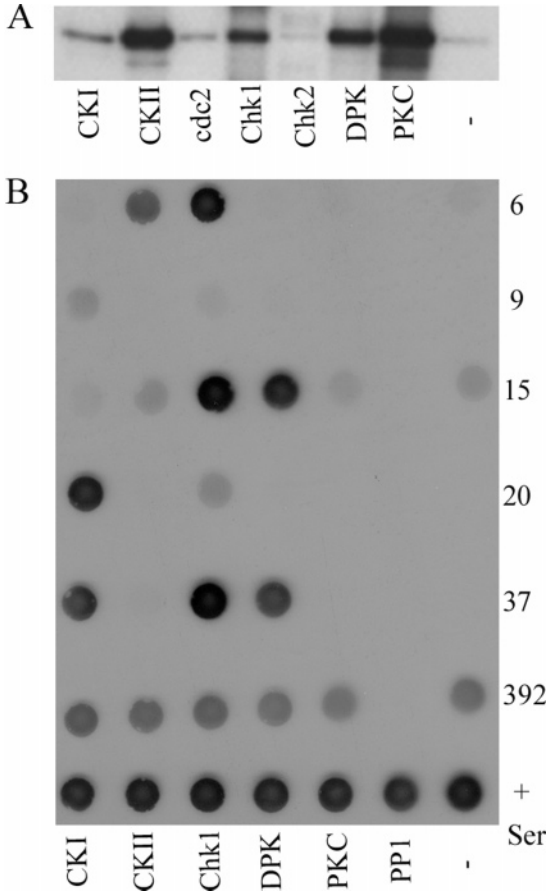

FIGURE 4: p53 phosphorylation by protein kinases. (A) Total phosphorylation of p53. Dephosphorylated p53 (90 nM) (see Experimental Procedures) was incubated with [γ-^{32}P]ATP and 200 units of CK1, CKII, cdc2, Chk1, Chk2, DNA-PK, or PKC for 20 min at room temperature. Products were analyzed by SDS−PAGE (10%) and autoradiography. (B) Site-specific phosphorylation of p53. Reactions were set up as described in (A), substituting the [γ-^{32}P]ATP with 25 μM unlabeled ATP, followed by immunoslot blot analysis using the phosphoserine antibodies. An antibody to total p53 (+) was used as a positive control.

same level as dephosphorylated protein (Figure 5 and Table 2). Treatment with DNA-PK resulted in supershifted complexes, suggesting cocomplexes between the kinase and p53. Quantification of the large smear showed that close to 90% of the input DNA was complexed with the proteins.

Binding of phosphorylated p53 to 3-cytosine bulge probes was also examined (Figure 6). CKI and PKC restored the original p53 binding activity while CKII had no effect, and complex formation was retained at the level of dephosphorylated protein. Like Holliday junction probes, a smear is seen with DNA-PK which probably represents cocomplexes between p53, DNA-PK, and the probe. Interestingly, CKI-treated p53 also caused supershifted complexes with the 3-cytosine bulge probe which were retained in the well. Presumably, like DNA-PK, these represent multimeric complexes of CKI, p53, and the probe.

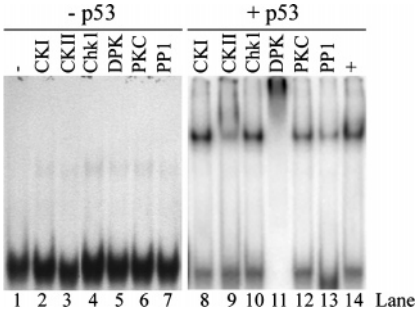

FIGURE 5: p53 binding to Holliday junctions following protein phosphorylation. Reactions containing no p53 (lanes 1−7) or dephosphorylated p53 (lanes 8−14) were incubated with various kinases in the presence of 25 μM unlabeled ATP as described in Figure 4 followed by the addition of end-labeled Holliday junctions for an additional 20 min. Reaction products were separated on 5% nondenaturing polyacrylamide gels followed by autoradiography.

Table 2[a]

protein	% probe bound
p53 (untreated)	48.5 ± 9.2
p53 (dephosphorylated)	19 ± 4.2
dephosphorylated p53 + PKC	40.5 ± 12
dephosphorylated p53 + DNA-PK	90% as smear
dephosphorylated p53 + Chk1	45.5 ± 2
dephosphorylated p53 + CKII	17 ± 4.2
dephosphorylated p53 + CKI	48 ± 10

[a] p53/Holliday junction complexes following kinase treatment were quantified as described in Experimental Procedures. Values represent data from three independent experiments.

While rephosphorylation of serine 392 was observed with all kinases, CKII was not able to restore binding activity, indicating the role of other residues in Holliday junction and lesion recognition. Collectively, the results show that the phosphorylation state of p53 plays a significant role in recognition of Holliday junctions and 3-cytosine bulges and that both sequence-dependent and -independent binding by p53 is strongly regulated by posttranslational modifications of the protein.

DISCUSSION

Recognition of unusual DNA structures represents an important function of p53 in addition to sequence-specific binding and transcriptional activation. To date, p53 has been shown to bind to a host of DNA structures including three- and four-way junctions, insertion/deletion mismatches, and

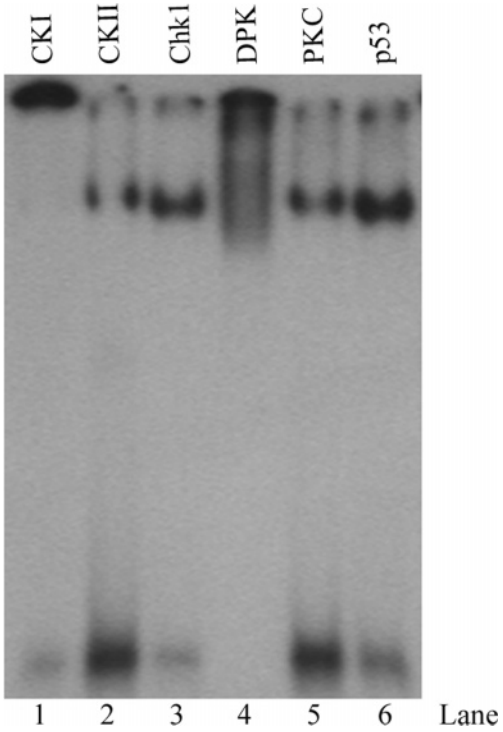

FIGURE 6: p53 binding to 3-cytosine bulges following protein phosphorylation. Dephosphorylated p53 was treated with various kinases in the presence of 25 μM unlabeled ATP as described in Figure 4 followed by the addition of end-labeled 3-cytosine bulges for an additional 20 min. Reaction products were separated on 5% nondenaturing polyacrylamide gels followed by autoradiography.

base/base mismatches as well as single and double strand breaks. Like its other functions, this activity appears to be regulated by several factors. We have previously shown that p53 recognition of Holliday junctions and 3-cytosine bulges is altered by other proteins such as HMG I/Y and hMSH2-hMSH6. HMG I/Y inhibits p53 binding to these lesions whereas hMSH2-hMSH6 promotes recognition (*12*). Here, we demonstrate that the phosphorylation state of p53 affects both sequence-dependent and -independent binding.

Loss of structure-specific binding with the removal of a C-terminal phosphate group (serine 392) with PP1 is consistent with the role of this domain in sequence-independent recognition. We have shown in a previous study that the C-terminal domain alone can bind insertion/deletion mismatches (*10*), and similarly there are other studies that illustrate the role of this domain in damage recognition (*9*, *46*−*48*). Removal of the phosphate group from this residue reduces binding to both Holliday junctions and 3-cytosine bulges. Interestingly, treatment with λP slightly reduces p53 complexes with Holliday junctions but not the 3-cytosine bulge probe, indicating that recognition of Holliday junctions is more sensitive to the posttranslational state of p53. This is similar to our previous findings that p53 complexes with Holliday junctions are more labile than the 3-cytosine bulges when HMG I/Y was added to the reactions (*12*). On the other hand, treatment with CKI results in supershifted complexes with the 3-cytosine bulges but not Holliday junctions. These results suggest that conformational changes in p53 induced by phosphorylation or binding of other proteins influence the mechanism by which p53 recognizes various structures. None of the other residues that were tested in this study seem to affect sequence-independent binding as removal of phosphates on serines 6 and 15 by λP did not inhibit recognition of 3-cytosine bulges and only slightly reduced Holliday junction binding.

The three kinases that restored DNA binding activity, CKI, Chk1, and PKC, were similar in their ability to rephosphorylate serine 392. Other residues were also phosphorylated individually by these kinases; e.g., CKI and Chk1 were also active on serine 37 but not PKC while CKI alone phosphorylated serine 20 and Chk1 alone modified serines 6 and 15. This suggests that serine 392 may play an important role in structure-specific binding. However, it is clear that other residues are also required as CKII was able to rephosphorylate serine 392 but was not able to restore binding activity. Since multiple sites were phosphorylated with the kinases used in this study, the exact modifications required are unclear. Interestingly, loss of the N-terminal phosphate groups (serines 6 and 15) alone disrupts sequence-specific p53 binding, indicating that the sequence-dependent and -independent binding are affected in different ways by posttranslational modifications.

Several reports have shown that CKII specifically phosphorylates serine 392 (*49*). However, unlike Chk1, which shows phosphate addition above the level of the native protein with serines 6, 15, and 37, CKII phosphorylation of serine 392 does not exceed that of untreated protein. p53 was treated with CKII using several different conditions, yet we were unable to achieve higher levels of phosphorylation of this residue. A more recent report has shown that CKII forms part of a larger complex consisting of hSpt16 and SSRP1 (*50*), which is responsible for the C-terminal phosphorylation, and the presence of these associated factors may be required to achieve full serine 392 modification. The possibility that this kinase is not active is negated by the fact that it was able to phosphorylate serine 6, an activity as yet unreported. Additionally, robust activity was seen when p53 was labeled with this kinase and [γ-^{32}P]ATP. We also find that CKI phosphorylates two novel sites, serines 20 and 37, which is, as yet, unreported.

Two of the kinases that restored structure-specific binding to p53, Chk1 and PKC, play important roles in the damage-response pathway. Chk1 has previously been shown to phosphorylate p53 at serines 15, 20, and 37 and acts downstream of ATR in response to UV irradiation (*26*, *51*). Its primary role is probably to increase protein stability; however, our data suggest that it also plays an important role in DNA structure recognition. Interestingly, no phosphorylation was seen with Chk2, which acts downstream of ATM in response to ionizing radiation (*52*). This correlates with a recent study from Ahn et al. (*53*), indicating that Chk2 is not a key regulator of p53. PKC has been shown to target the C-terminal domain of p53 and plays a role in activating p53 sequence-specific binding (*35*). Our results further confirm the importance of this kinase in p53 posttranslational modifications as well as structure-specific binding.

The exact role of DNA-PK in the p53 response pathways is unclear, and in vivo, phosphorylation by this kinase is not required for transcription activation or cell cycle arrest mediated by p53 (*54*, *55*). In a recent study by Dip et al. (*56*), the authors showed by photo-cross-linking experiments that DNA-PK binds to Holliday junctions which we did not detect in our assays. However, in these studies binding of DNA-PK to Holliday junctions but not its phosphorylation

activity was inhibited by the addition of KCl and ATP, both of which are present in our reaction conditions. While direct binding of DNA-PK to Holliday junctions was not observed in our study, supershifted complexes were seen in reactions containing both p53 and DNA-PK, suggesting that recruitment of this kinase to Holliday junctions may require protein−protein interactions with p53. This would be similar to the effect of Ku on the affinity of DNA-PK for DNA termini (*57*). Alternately, under the appropriate conditions, DNA-PK binding to Holliday junctions could serve as a mechanism of p53 recruitment to sites of structurally altered DNA by phosphorylation events, which fits well with its role in detection and repair to DNA damage.

In the cellular environment, it is likely that multiple kinases will act on p53 following DNA damage, and it is the collective effect of these phosphorylation events that will control downstream activation of the protein. Overall, it is clear that phosphorylation events dictate p53 binding activities, both sequence dependent and independent, and these modifications guide p53 to the appropriate sites of binding. To further elucidate key residues involved in structure-specific binding, p53 mutants containing changes in single amino acids, especially serine 392, need to be characterized. The role of structure-specific recognition in downstream pathways is not clear. The decision to evoke cell cycle arrest versus apoptotic pathways must, to some extent, be determined by the amount of damage that is present in the cell. If this is the case, then p53 would be required to survey the amount and type of DNA alterations that have occurred, thus facilitating recruitment of repair proteins to the appropriate sites. Alternately, binding to these structures may itself assist in transactivation of gene products involved in the cell cycle arrest or apoptotic pathways. Indeed, recent studies argue against allosteric regulation of putative genes in vivo (*58*); thus damage recognition may the more predominant pathway of invoking downstream functions.

ACKNOWLEDGMENT

We thank members of the Griffith laboratory for helpful discussions.

REFERENCES

1. Maltzman, W., and Czyzyk, L. (1984) UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells, *Mol. Cell. Biol. 4*, 1689−1694.
2. Price, B. D., and Calderwood, S. K. (1993) Increased sequence-specific p53-DNA binding activity after DNA damage is attenuated by phorbol esters, *Oncogene 8*, 3055−3062.
3. Maki, C. G., and Howley, P. M. (1997) Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation, *Mol. Cell. Biol. 17*, 355−363.
4. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R. W. (1991) Participation of p53 protein in the cellular response to DNA damage, *Cancer Res. 51*, 6304−6311.
5. Meek, D. W. (1999) Mechanisms of switching on p53: a role for covalent modification?, *Oncogene 18*, 7666−7675.
6. Woods, D. B., and Vousden, K. H. (2001) Regulation of p53 function, *Exp. Cell Res. 264*, 56−66.
7. Ko, L. J., and Prives, C. (1996) p53: puzzle and paradigm, *Genes Dev. 10*, 1054−1072.
8. Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division, *Cell 88*, 323−331.
9. Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K. P., Szekely, L., Klein, G., Terenius, L., and Wiman, K. G. (1995) p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain, *Nucleic Acids Res. 23*, 362−369.
10. Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995) p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches, *Cell 81*, 1013−1020.
11. Lee, S., Cavallo, L., and Griffith, J. (1997) Human p53 binds Holliday junctions strongly and facilitates their cleavage, *J. Biol. Chem. 272*, 7532−7539.
12. Subramanian, D., and Griffith, J. D. (2002) Interactions between p53, hMSH2-hMSH6 and HMG I(Y) on Holliday junctions and bulged bases, *Nucleic Acids Res. 30*, 2427−2434.
13. Dudenhoffer, C., Rohaly, G., Will, K., Deppert, W., and Wiesmuller, L. (1998) Specific mismatch recognition in heteroduplex intermediates by p53 suggests a role in fidelity control of homologous recombination, *Mol. Cell. Biol. 18*, 5332−5342.
14. Degtyareva, N., Subramanian, D., and Griffith, J. D. (2001) Analysis of the binding of p53 to DNAs containing mismatched and bulged bases, *J. Biol. Chem. 276*, 8778−8784.
15. Appella, E., and Anderson, C. W. (2001) Post-translational modifications and activation of p53 by genotoxic stresses, *Eur. J. Biochem. 268*, 2764−2772.
16. Persons, D. L., Yazlovitskaya, E. M., and Pelling, J. C. (2000) Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin, *J. Biol. Chem. 275*, 35778−35785.
17. Ashcroft, M., Taya, Y., and Vousden, K. H. (2000) Stress signals utilize multiple pathways to stabilize p53, *Mol. Cell. Biol. 20*, 3224−3233.
18. Webley, K., Bond, J. A., Jones, C. J., Blaydes, J. P., Craig, A., Hupp, T., and Wynford-Thomas, D. (2000) Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage, *Mol. Cell. Biol. 20*, 2803−2808.
19. Ferbeyre, G., de Stanchina, E., Querido, E., Baptiste, N., Prives, C., and Lowe, S. W. (2000) PML is induced by oncogenic ras and promotes premature senescence, *Genes Dev. 14*, 2015−2027.
20. Kapoor, M., and Lozano, G. (1998) Functional activation of p53 via phosphorylation following DNA damage by UV but not gamma radiation, *Proc. Natl. Acad. Sci. U.S.A. 95*, 2834−2837.
21. Appella, E., and Anderson, C. W. (2000) Signaling to p53: breaking the posttranslational modification code, *Pathol. Biol. (Paris) 48*, 227−245.
22. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage, *Science 281*, 1674−1677.
23. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Activation of the ATM kinase by ionizing radiation and phosphorylation of p53, *Science 281*, 1677−1679.
24. Khanna, K. K., Keating, K. E., Kozlov, S., Scott, S., Gatei, M., Hobson, K., Taya, Y., Gabrielli, B., Chan, D., Lees-Miller, S. P., and Lavin, M. F. (1998) ATM associates with and phosphorylates p53: mapping the region of interaction, *Nat. Genet. 20*, 398−400.
25. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) A role for ATR in the DNA damage-induced phosphorylation of p53, *Genes Dev. 13*, 152−157.
26. Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000) The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites, *Genes Dev. 14*, 289−300.
27. Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998) DNA damage activates p53 through a phosphorylation-acetylation cascade, *Genes Dev. 12*, 2831−2841.
28. Dumaz, N., and Meek, D. W. (1999) Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2, *EMBO J. 18*, 7002−7010.
29. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. (1992) Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53, *Mol. Cell. Biol. 12*, 5041−5049.
30. Milne, D. M., Palmer, R. H., Campbell, D. G., and Meek, D. W. (1992) Phosphorylation of the p53 tumour-suppressor protein at three N-terminal sites by a novel casein kinase I-like enzyme, *Oncogene 7*, 1361−1369.

31. Milne, D. M., Campbell, D. G., Caudwell, F. B., and Meek, D. W. (1994) Phosphorylation of the tumor suppressor protein p53 by mitogen-activated protein kinases, *J. Biol. Chem. 269*, 9253−9260.
32. Bischoff, J. R., Friedman, P. N., Marshak, D. R., Prives, C., and Beach, D. (1990) Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2, *Proc. Natl. Acad. Sci. U.S.A. 87*, 4766−4770.
33. Price, B. D., Hughes-Davies, L., and Park, S. J. (1995) Cdk2 kinase phosphorylates serine 315 of human p53 in vitro, *Oncogene 11*, 73−80.
34. Wang, Y., and Prives, C. (1995) Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases, *Nature 376*, 88−91.
35. Takenaka, I., Morin, F., Seizinger, B. R., and Kley, N. (1995) Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases, *J. Biol. Chem. 270*, 5405−5411.
36. Hupp, T. R., Meek, D. W., Midgley, C. A., and Lane, D. P. (1992) Regulation of the specific DNA binding function of p53, *Cell 71*, 875−886.
37. Muller, S., Berger, M., Lehembre, F., Seeler, J. S., Haupt, Y., and Dejean, A. (2000) c-Jun and p53 activity is modulated by SUMO-1 modification, *J. Biol. Chem. 275*, 13321−13329.
38. Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999) SUMO-1 modification activates the transcriptional response of p53, *EMBO J. 18*, 6455−6461.
39. Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999) Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1, *EMBO J. 18*, 6462−6471.
40. Prives, C., and Hall, P. A. (1999) The p53 pathway, *J. Pathol. 187*, 112−126.
41. Smith, M. L., Ford, J. M., Hollander, M. C., Bortnick, R. A., Amundson, S. A., Seo, Y. R., Deng, C. X., Hanawalt, P. C., and Fornace, A. J., Jr. (2000) p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes, *Mol. Cell. Biol. 20*, 3705−3714.
42. Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J., and McKay, B. C. (1999) Inhibition of RNA polymerase II as a trigger for the p53 response, *Oncogene 18*, 583−592.
43. Wu, L., Bayle, J. H., Elenbaas, B., Pavletich, N. P., and Levine, A. J. (1995) Alternatively spliced forms in the carboxy-terminal domain of the p53 protein regulate its ability to promote annealing of complementary single strands of nucleic acids, *Mol. Cell. Biol. 15*, 497−504.
44. Patterson, R. M., He, C., Selkirk, J. K., and Merrick, B. A. (1996) Human p53 expressed in baculovirus-infected Sf9 cells displays a two-dimensional isoform pattern identical to wild-type p53 from human cells, *Arch. Biochem. Biophys. 330*, 71−79.
45. Merrick, B. A., Zhou, W., Martin, K. J., Jeyarajah, S., Parker, C. E., Selkirk, J. K., Tomer, K. B., and Borchers, C. H. (2001) Site-specific phosphorylation of human p53 protein determined by mass spectrometry, *Biochemistry 40*, 4053−4066.
46. Bayle, J. H., Elenbaas, B., and Levine, A. J. (1995) The carboxyl-terminal domain of the p53 protein regulates sequence-specific DNA binding through its nonspecific nucleic acid-binding activity, *Proc. Natl. Acad. Sci. U.S.A. 92*, 5729−5733.
47. Reed, M., Wang, Y., Mayr, G., Anderson, M. E., Schwedes, J. F., and Tegtmeyer, P. (1993) p53 domains: suppression, transformation, and transactivation, *Gene Expression 3*, 95−107.
48. Wang, N. P., To, H., Lee, W. H., and Lee, E. Y. (1993) Tumor suppressor activity of RB and p53 genes in human breast carcinoma cells, *Oncogene 8*, 279−288.
49. Meek, D. W., Simon, S., Kikkawa, U., and Eckhart, W. (1990) The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II, *EMBO J. 9*, 3253−3260.
50. Keller, D. M., Zeng, X., Wang, Y., Zhang, Q. H., Kapoor, M., Shu, H., Goodman, R., Lozano, G., Zhao, Y., and Lu, H. (2001) A DNA damage-induced p53 serine 392 kinase complex contains CK2, hSpt16, and SSRP1, *Mol. Cell 7*, 283−292.
51. Chehab, N. H., Malikzay, A., Stavridi, E. S., and Halazonetis, T. D. (1999) Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage, *Proc. Natl. Acad. Sci. U.S.A. 96*, 13777−13782.
52. Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000) DNA damage-induced activation of p53 by the checkpoint kinase Chk2, *Science 287*, 1824−1827.
53. Ahn, J., Urist, M., and Prives, C. (2003) Questioning the role of checkpoint kinase 2 in the p53 DNA damage response, *J. Biol. Chem. 278*, 20480−20489.
54. Araki, R., Fukumura, R., Fujimori, A., Taya, Y., Shiloh, Y., Kurimasa, A., Burma, S., Li, G. C., Chen, D. J., Sato, K., Hoki, Y., Tatsumi, K., and Abe, M. (1999) Enhanced phosphorylation of p53 serine 18 following DNA damage in DNA-dependent protein kinase catalytic subunit-deficient cells, *Cancer Res. 59*, 3543−3546.
55. Jimenez, G. S., Bryntesson, F., Torres-Arzayus, M. I., Priestley, A., Beeche, M., Saito, S., Sakaguchi, K., Appella, E., Jeggo, P. A., Taccioli, G. E., Wahl, G. M., and Hubank, M. (1999) DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage, *Nature 400*, 81−83.
56. Dip, R., and Naegeli, H. (2004) Binding of the DNA-dependent protein kinase catalytic subunit to Holliday junctions, *Biochem. J. 381*, 165−174.
57. Hammarsten, O., and Chu, G. (1998) DNA-dependent protein kinase: DNA binding and activation in the absence of Ku, *Proc. Natl. Acad. Sci. U.S.A. 95*, 525−530.
58. Kaeser, M. D., and Iggo, R. D. (2002) Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo, *Proc. Natl. Acad. Sci. U.S.A. 99*, 95−100.

BI048700U