

# A DNA Damage–Induced p53 Serine 392 Kinase Complex Contains CK2, hSpt16, and SSRP1

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## Summary

Phosphorylation of the human p53 protein at Ser-392 has been shown to be responsive to UV but not  $\gamma$  irradiation. Here we describe identification and purification of a mammalian UV-activated protein kinase complex that phosphorylates Ser-392 of p53 *in vitro*. This kinase complex contains casein kinase 2 (CK2) and the chromatin transcriptional elongation factor FACT (a heterodimer of hSpt16 and SSRP1). *In vitro* studies show that FACT alters the specificity of CK2 in the complex such that it selectively phosphorylates p53 over other substrates including casein. In addition, phosphorylation by the kinase complex enhances p53 activity. These results thus provide a potential mechanism for p53 activation by UV irradiation.

## Introduction

The tumor suppressor protein p53 is targeted by a wide variety of intracellular and extracellular stimuli (Levine, 1997; Giaccia and Kastan, 1998; Oren and Rotter, 1999). One group of stimuli includes genotoxic agents, such as irradiation, chemicals, and oxygen-free radicals (Tishler et al., 1993; Forrester et al., 1996; Lu et al., 1998; Hirao et al., 2000). Others include withdrawal of growth factors (Gottlieb et al., 1994), oncogenes (Lowe and Riley, 1993; Serrano et al., 1997), hypoxia (Graeber et al., 1994), and defects in nucleotide synthesis (Linke et al., 1996). As a consequence of these stresses, p53 is stabilized at the protein level, and its transcriptional activity is enhanced (Lakin and Jackson, 1999). This activation of p53 leads, primarily through its transcriptional function (Unger et al., 1993), to either apoptosis, eliminating those cells harboring severely damaged DNA, or growth arrest, allowing damaged DNA to be repaired and thereby suppressing tumor formation.

Stability and activity of p53 are believed to be regulated in part by posttranslational modifications, such

as phosphorylation and acetylation (Shieh et al., 1997; Sakaguchi et al., 1998; Lakin and Jackson, 1999; Unger et al., 1999). These modifications allow rapid modulation of p53 function in response to specific stimuli, as different subsets of modifications may result depending on the inducing conditions (Buschmann et al., 2000). Probably the best-characterized example of this is following ultraviolet (UV) or  $\gamma$  irradiation, which both cause DNA damage but activate distinct DNA repair pathways (Lindahl and Wood, 1999). After  $\gamma$  irradiation, several serine residues on both the N and C termini of p53 are targeted, possibly due to the action of the  $\gamma$ -responsive ataxia telangiectasia mutated (ATM) kinase (Banin et al., 1998; Waterman et al., 1998). This is strikingly demonstrated in A-T cells, which contain nonfunctional ATM, where p53 upregulation is defective after  $\gamma$  irradiation (Kastan et al., 1992; Khanna and Lavin, 1993). Recent studies have shown that ATM may not directly phosphorylate p53, but instead may act through its downstream kinase, hChk2, which targets Ser-20 (Bell et al., 1999; Hirao et al., 2000). Phosphorylation at this site is known to impede binding of the p53 negative regulator, MDM2, thus increasing p53 stability (Chehab et al., 1999; Unger et al., 1999).

Phosphorylation of human p53 Ser-392 (corresponding to murine Ser-389; for simplicity, Ser-392 will be used) in the C-terminal regulatory domain occurs following UV but not  $\gamma$  irradiation (Kapoor and Lozano, 1998; Lu et al., 1998) and results in enhancement of sequence-specific binding activity *in vitro* (Hupp et al., 1992), possibly by promoting stable tetramer formation (Sakaguchi et al., 1997). Substitution of Ser-392 with an acidic residue constitutively activates p53 as a transcription factor (Hao et al., 1996). The importance of Ser-392 in regulating p53 function is also reflected by the fact that this residue is evolutionarily conserved from humans to bony fishes (Figure 1). However, the kinase(s) that targets this residue *in vivo* has not been elucidated conclusively. Previously, the protein kinase CK2 was shown to target this site *in vitro* (Meek et al., 1990). Recently, phosphorylation at this site *in vivo* was shown to be inhibited by p38MAPK-specific inhibitors (Huang et al., 1999; Keller et al., 1999), suggesting that p38MAPK may target this serine directly or indirectly. Interestingly, p38MAPK was also reported to phosphorylate and activate CK2 after DNA damage (Sayed et al., 2000), although it is yet unclear whether a p38MAPK-CK2-p53 pathway exists in cells.

To investigate the UV-responsive signaling pathway to p53 via Ser-392, we have identified and purified a DNA damage responsive kinase activity that targets this residue from murine embryonic testicular carcinoma F9 or human cervical carcinoma HeLa nuclear extracts. This kinase contains seven polypeptides with a native molecular weight of  $\sim 700$  kDa, including the CK2 subunits, and the two subunits of the human FACT (facilitates chromatin transcription) complex, hSpt16/Cdc68 and SSRP1 (structure-specific recognition protein-1) (Orphanides et al., 1999). This p53 Ser-392 kinase com-

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Ser392 is evolutionarily conserved

|                     |   |   |   |   |   |   |   |   |       |
|---------------------|---|---|---|---|---|---|---|---|-------|
| Human:              | K | T | E | G | P | D | S | D | (393) |
| Rhesus Monkey:      | K | T | E | G | P | D | S | D | (393) |
| Mouse:              | K | K | V | G | P | D | S | D | (390) |
| Rat:                | K | K | V | G | P | D | S | D | (390) |
| Cat:                | K | R | E | G | L | D | S | D | (376) |
| Pig:                | K | R | E | G | P | D | S | E | (376) |
| Xenopus:            | K | D | E | Q | P | D | S | E | (363) |
| Rainbow Trout:      | K | E | E | K | S | D | S | D | (396) |
| Platichthys flesus: | K | G | E | R | S | D | S | D | (366) |
| Chicken:            | K | L | L | Q | K | G | S | D | (367) |

Figure 1. Ser-392 Is Evolutionarily Conserved  
Sequence alignment of carboxy-terminal amino acids of p53, showing that Ser-392 of human p53 is conserved through mammals, amphibians, bony fishes, and birds. Numbers in parentheses refer to the last amino acid residue of p53 for each species.

plex preferentially phosphorylates Ser-392 over other CK2 substrates and stimulates p53 activity.

Results

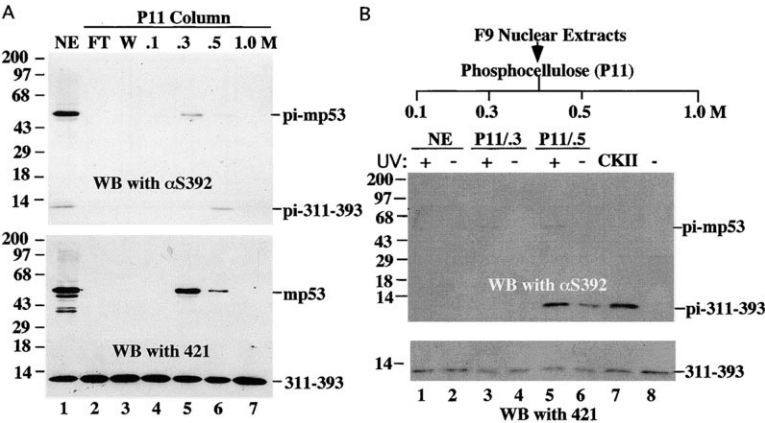
**Identification of a UV-Responsive p53 Ser-392 Kinase**  
Our previous studies showed that Ser-392 phosphorylation is responsive to UV but not  $\gamma$  irradiation (Kapoor and Lozano, 1998; Lu et al., 1998). This appears to be a general phenomenon, as it occurred in all the p53-containing cell lines tested (data not shown). To identify the UV-responsive p53 Ser-392 kinase, we performed a fractionation starting with nuclear extracts from F9 cells. This cell line was chosen because it contains wild-type p53 and shows a strong p53 response to DNA damage caused by 20 J/m<sup>2</sup> UVC (Lu et al., 1998). Cells were treated with or without 20 J/m<sup>2</sup> UVC and harvested 8 hr later. Nuclear extracts were prepared and fractionated on a phosphocellulose (P11) column (Figure 2A). An in vitro kinase assay was performed on the column fractions using unlabeled ATP and a C-terminal fragment of p53 (amino acids 311–393) as substrates. Phosphorylated p53 was detected by Western blot (WB) using a Ser-392 phospho-specific antibody (Kapoor and Lozano, 1998). Kinase activity was detected in the 0.5 M

KCl fraction (top panel, lane 6), while endogenous p53 was detected largely in the 0.35 M KCl fraction (lane 5) and residually in the 0.5 M KCl fraction (lane 6). Equal levels of the 311–393 peptide were added as determined by WB with PAb421, a p53 antibody that recognizes a C-terminal epitope (bottom panel). This p53 Ser-392 kinase activity was stimulated after UV irradiation, as shown in Figure 2B (compare lane 5 with lane 6). CK2 alone was used as a positive control (lane 7). p38MAPK eluted in the 0.1 M KCl fraction as detected by WB (data not shown) and so did not appear to be responsible for this phosphorylation. Thus, we detected UV-responsive p53 Ser-392 kinase activity in F9 cells.

Purification of the p53 Ser-392 Kinase

We did not obtain sufficient amounts of protein from F9 cells for the final purification step, so we repeated the purification using HeLa cells, which can be cultured in large volumes. As in F9 cells, p53 Ser-392 kinase activity from HeLa cells was detected solely in the P11 0.5 M KCl fraction (data not shown). Furthermore, all subsequent purification steps showed identical p53 Ser-392 kinase activity profiles between HeLa and F9 cells. Purification was carried out through several steps (Figure 3A), and the fold purification was judged to be 40,900 (Table 1). p53 Ser-392 kinase activity from a representative column (MonoQ) was analyzed using a radioactive kinase assay and a WB kinase assay using the anti-Ser-392 phospho-specific antibody (Figure 3B). The result from this analysis clearly demonstrated the specificity of the anti-phosphorylated Ser-392 antibody, as kinase activity targeting non-Ser-392 residues was separated from the p53 Ser-392 kinase (compare the top panel with the middle panel).

To determine the native molecular weight of the kinase, a Superdex 200 gel filtration column was used as the last step in the purification (Figure 4). Kinase activity eluted at a native molecular weight of close to 670 kDa



(B) p53 Ser-392 kinase activity in the P11 0.5 M KCl fraction is stimulated after UV irradiation. F9 nuclear extracts from both UV-treated and untreated cells were run on a P11 column. A cold kinase assay using the various fractions (1  $\mu$ g protein) was performed identically as in (A).

Figure 2. p53 Ser-392 Kinase Activity Is Stimulated by UV  
(A) p53 Ser-392 kinase activity is present in the 0.5 M KCl fraction. F9 cells were treated with 20 J/m<sup>2</sup> UVC for 8 hr and harvested for nuclear extract preparation. Extracts (from 10<sup>8</sup> cells) were loaded onto a phosphocellulose (P11) column, and fractions were collected at the indicated KCl concentrations. In vitro cold kinase assays were performed using 1  $\mu$ g of the indicated fraction and unlabeled ATP and 100 ng p53 C-terminal peptide (aa 311–393) as substrates. Phosphorylated products were detected by Western blot with a phospho-specific Ser-392 antibody (top panel), while WB with PAb421 was used to show equal loading of substrates (bottom panel). Endogenous murine p53 is denoted by mp53, and the phosphorylated form is denoted by pi-mp53.

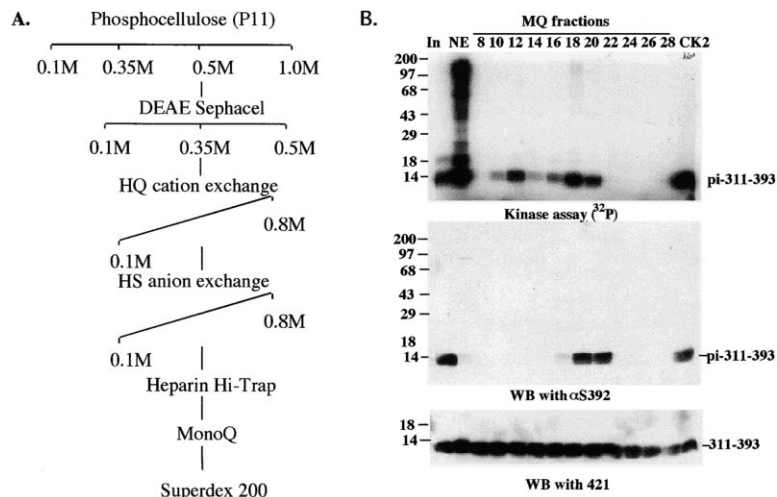


Figure 3. Purification Scheme of p53 Ser-392 Kinase

(A) p53 Ser-392 kinase purification procedure. To scale up the kinase purification, HeLa cells ( $10^{10}$  cells) were employed. p53 Ser-392 kinase activity was monitored through each fractionation step by kinase assays as described in Experimental Procedures. See the legend for Table 1 for details. (B) Anti-Ser-392 antibodies selectively detected p53 Ser-392 kinase activity in the MonoQ fractions. Column fractions (3  $\mu$ l) were used in a radioactive kinase assay (top panel) and a WB kinase assay using the anti-Ser-392 antibody (middle panel). C-terminal p53 311–393 (100 ng) detected by Pab421 was used as a substrate in each reaction (bottom panel).

(Figures 4A and 4B), suggesting that the kinase is part of a protein complex. Indeed, colloidal blue staining analysis of the active proteins pooled from fractions 9 to 11 showed that this kinase complex is composed of at least seven polypeptides (Figure 4C). p400 might not be part of the complex, as it did not appear in a separate purification (data not shown). Mass spectrometry revealed the identity of five of the polypeptides (see Experimental Procedures). p140 was identified as hSpt16, p80 as SSRP1, p41 as CK2 $\alpha'$ , p37 as CK2 $\alpha$ , and p27 as CK2 $\beta$ . The identities of the other polypeptides are currently unknown because the amount obtained of these large proteins was insufficient for mass spectrometry. These results indicate that CK2 can associate with the transcriptional elongation factor FACT (hSpt16 and SSRP1) to form a large kinase complex.

#### Association of hSpt16 and SSRP1 with CK2

The above results (Figure 4) suggest that CK2 might associate with the FACT heterodimer that was recently identified as a transcriptional elongation factor (Orphanides et al., 1999). To test this, CK2, hSpt16, and SSRP1 were traced throughout a subsequent purification by WB using antibodies against these proteins (Figure 5A). We observed that these proteins cofractionated through all the columns used for purification (see Figure 3A for procedures). As shown in one representative WB result from a MonoQ column (Figure 5A), CK2 $\alpha'$  and  $\beta$

subunits copurified with hSpt16 and SSRP1, as well as with p53 Ser-392 kinase activity, peaking at fraction 17. Consistent with this result and the results in Figure 4, CK2  $\alpha'$  and p53 Ser-392 kinase activity were coimmunoprecipitated from the P11 0.5 M KCl fraction by the affinity-purified polyclonal anti-hSpt16 and anti-SSRP1 antibodies, but not rabbit IgG (Figure 5B). To investigate how abundant the CK2-hSpt16-SSRP1 complex was in the cell, gel filtration analysis was performed on a crude fraction from the purification procedure (P11 0.5 M KCl) (data not shown). We estimate that  $\sim$ 5% of hSpt16 and SSRP1 in the cell is associated with CK2 in the high molecular weight complex, while the majority is free of CK2. From these results, we conclude that CK2 indeed associates with hSpt16 and SSRP1.

#### The p53 Ser-392 Kinase Complex and CK2 Alone Display Distinct Substrate Specificities

The finding that the purified p53 Ser-392 kinase complex contains CK2 prompted us to test whether this complex shares the same substrates with CK2 alone. To this end, kinase assays were performed using several known CK2 substrates, including casein, GST- $\Delta$ MDM2, the p53 C terminus (aa 311–393), or histone H1 (Figure 6A). As expected, CK2 (Promega) phosphorylated all the tested substrates, with a strong preference for casein and the p53 C-terminal fragment. Surprisingly, the purified kinase containing CK2, hSpt16, and SSRP1 preferentially

Table 1. Fold Purification of p53 Ser-392 Kinase

| Fraction            | Protein (mg) | Kinase Units (Western) | Specific Activity (U/mg) | Fold Purification |
|---------------------|--------------|------------------------|--------------------------|-------------------|
| NE                  | 430          | 6,080                  | 14                       | 1                 |
| P <sub>11</sub> 0.5 | 24           | 30,700                 | 1,280                    | 91                |
| DE 0.35             | 14           | 87,600                 | 6,260                    | 450               |
| HQ pool             | 4.1          | 129,000                | 31,500                   | 2,250             |
| HS pool             | 1.2          | 120,000                | 100,000                  | 7,140             |
| S200 frac.10        | 0.0025       | 1,430                  | 572,000                  | 40,900            |

Fractions from purification steps were used in an in vitro kinase assay using the phospho-specific  $\alpha$ Ser-392 antibody to detect phosphorylated product. Following Western blot, the visualized bands were quantified by densitometry using BioRad Molecular Analyst software. NE, nuclear extract; P<sub>11</sub> 0.5, phosphocellulose 0.5 M KCl fraction; DE 0.35, DEAE Sephacel 0.35 M KCl fraction; HQ pool, HQ 4.6/100 column active fractions; HS pool, HS 4.6/100 column active fractions; S200 frac. 10, Superdex 200 gel filtration column fraction 10.

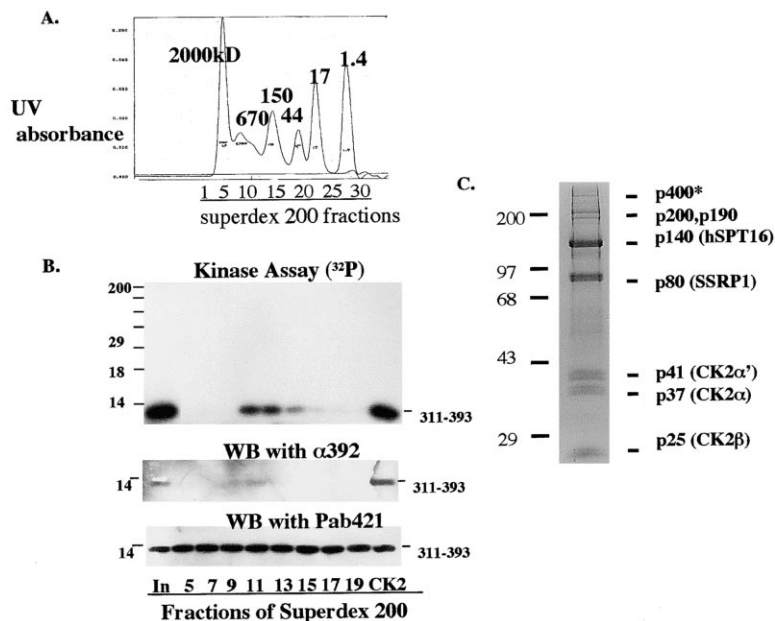


**Figure 4. p53 Ser-392 Kinase Is Associated with a High Molecular Weight Protein Complex**  
(A) Chromatograph of molecular weight standards loaded on a Superdex 200 column.  
(B) p53 Ser-392 kinase activity elutes at a native molecular weight of 700 kDa as judged by both a radioactive kinase assay using [ $\gamma$ - $^{32}$ P]ATP (top) and a Western blot kinase assay (middle). Column fractions (3  $\mu$ l) were used in each lane with 100 ng C-terminal p53 311–393 (detected by Pab421 in bottom panel).  
(C) Fractions 9–11 (7.5  $\mu$ g protein) were pooled and subjected to SDS-PAGE and stained with colloidal blue, followed by digestion for mass spectrometry. Corresponding proteins identified by MS analysis were labeled on the right of the panel. Asterisk denotes the possible contaminating protein, as it did not show in a separate purification.

targeted the p53 fragment, with little activity on casein (compare lanes 2 and 3 with lanes 5 and 6). Also, this kinase complex did not phosphorylate GST- $\Delta$ MDM2 and histone H1 as efficiently as CK2 alone (compare lanes 8, 9, 11, and 12 with lanes 5 and 6). This discrepancy between the p53 Ser-392 kinase complex and CK2 alone was not caused by different amounts or activity of each kinase used in this assay, as both of the kinases showed equivalent activity on the p53 C-terminal fragment (lanes 2 and 3).

These results suggested that CK2 preferentially phosphorylates p53 when complexed together with hSpt16 and SSRP1. To confirm this, we attempted to reconstitute the substrate selectivity in vitro using purified components of the p53 Ser-392 kinase complex. As shown in Figure 6B, CK2 alone phosphorylated substrates 311–393 (C-terminal p53) and casein equally well (compare lanes 1 and 8). However, addition of either recombinant his-SSRP1 (lanes 2 and 3) or Flag-hSpt16 alone (lanes 4 and 5), or both together (lanes 6 and 7) caused a dramatic reduction in casein phosphorylation. One hundred nanograms of both proteins together led to a complete inhibition of casein phosphorylation. In stark contrast, 311–393 phosphorylation was enhanced by either his-SSRP1 (lanes 9 and 10) or Flag-hSpt16 (lanes 11 and 12), or both together (lanes 13 and 14). Likewise, full-length p53 is also a preferential target for the reconstituted kinase complex (Figure 6C). Furthermore, the effect of hSpt16 and SSRP1 is specific, because an N-terminal truncation mutant of SSRP1 cannot reproduce the effect of the full-length protein (Figure 6C, lanes 5 and 6). Additionally, we observed that SSRP1 itself is an efficient substrate for CK2 in vitro, while hSpt16 was an inefficient substrate (see Figure 6B). In Figure 6C, phosphorylation of SSRP1 was not visualized because less protein was added (25 ng compared to 100 ng in Figure 6B). These results demonstrate that CK2, while part of the p53 Ser-392 kinase complex, preferentially targets p53 over the other substrates tested.

#### Phosphorylation by the p53 Ser-392 Kinase Complex Enhances p53 Function

Phosphorylation at Ser-392 is known to enhance sequence-specific DNA binding in vitro (Hupp et al., 1992). To test the functional consequence of phosphorylation of p53 by the purified kinase, gel mobility shift analysis was performed (Figures 7A and 7B). In vitro kinase assays were carried out with recombinant full-length p53 and with or without ATP while the purified kinase was titrated. The kinase mixtures were then added to a DNA binding cocktail containing a radiolabeled DNA probe with a p53 binding site from the p21waf1/cip1 promoter. Adding the purified kinase alone without ATP enhanced p53 binding (compare lane 2 with 3, 5, and 7 of Figure 7A), suggesting a factor is present that intrinsically promotes DNA binding. This factor may be SSRP1, because it was recently shown to stimulate sequence-specific DNA binding activity of another transcription factor (Spencer et al., 1999). Addition of ATP to the reaction further stimulated DNA–p53 complex formation (compare lanes 3, 5, and 7 with 4, 6, and 8), suggesting that phosphorylation at Ser-392 by this kinase does indeed have a functional significance. The shifted bands were truly p53–DNA complexes because inclusion of a p53 antibody, 1801, resulted in a supershift of this complex (Figure 7B, lane 6). Similarly, competition with an unlabeled p53RE-containing competitor, but not with poly(dIdC), completely eliminated the shifted signal (compare lanes 4 and 5).

To determine whether enhanced DNA binding translates to more efficient p53-dependent transcription, an in vitro transcription assay was performed (Figure 7C). WT or S392A mutant p53 were incubated with or without ATP and the purified kinase and then added into a reconstituted transcription system as described in Experimental Procedures. The p53 Ser-392 kinase complex stimulated transcription from a promoter containing a p53 binding element, and the stimulation was dependent upon WT but not S392A mutant p53. In the same fashion

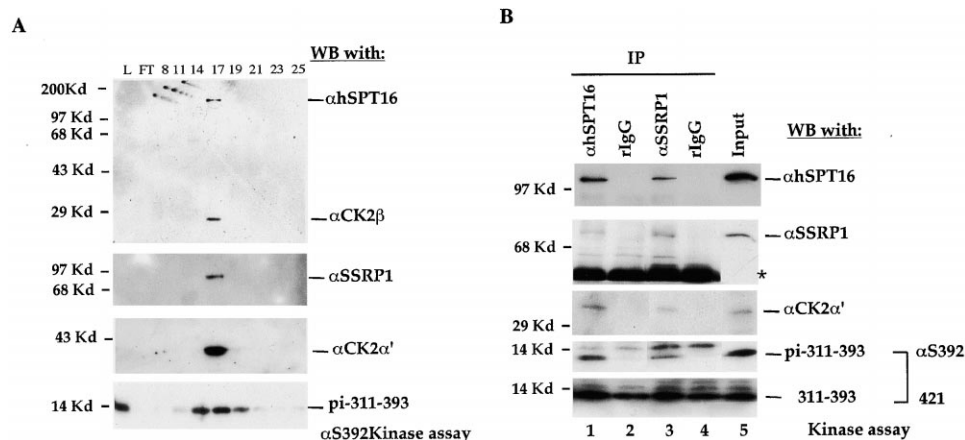


Figure 5. hSpt16 and SSRP1 Associate with CK2

(A) Representative column from the purification (MonoQ) showing coelution of CK2 $\alpha'$  and  $\beta$  subunits with hSpt16 and SSRP1. Column fractions (15  $\mu$ l) were run on SDS-PAGE for Western blot with the indicated antibodies. A Western blot kinase assay was performed as in the bottom panel and demonstrates that p53 Ser-392 kinase activity peak coelutes with these proteins.

(B) Immunoprecipitation (IP) of CK2 and p53 Ser-392 kinase activity with hSpt16 and SSRP1. IPs were done using either normal rabbit IgG,  $\alpha$ hSpt16, or  $\alpha$ SSRP1 antibodies (1  $\mu$ g each) to pull down the members of the complex as indicated from the P11 0.5 M KCl fraction. These same IPs were used in a Western blot kinase assay to pull down p53 Ser-392 kinase activity using 311–393 as a substrate. Asterisk indicates IgG heavy chain.

as the gel mobility shift assay, preincubating WT p53 with the p53 Ser-392 kinase complex without ATP stimulated RNA transcript levels (lane 6), but a 4- to 5-fold increase was achieved with inclusion of ATP (lane 7). Thus, phosphorylation of p53 at Ser-392 enhances p53's sequence-specific DNA binding and transcriptional activity in vitro.

Finally, we wished to test what effect phosphorylation of Ser-392 had on p53 function in vivo. To this end, we performed a luciferase assay comparing the transcriptional abilities of WT murine p53 and a S389A mutant (Figure 7D). p53 null H1299 cells were transiently transfected with either WT or S389A mutant p53, as well as a p53RE-luciferase construct derived from the p21waf1/cip1 promoter and  $\beta$ -galactosidase as an internal control. Forty-eight hours after transfection, the cells were UV-treated and harvested 1 or 2 hr after treatment. As shown in the graph, p53-dependent luciferase activity increased 2- to 3-fold following UV irradiation in the cells ectopically expressing WT but not mutant p53. IPs were done with PAb 421 and probed with the anti-Ser-392 or anti-p53 polyclonal antibodies. Phosphorylation after UV treatment correlated well with enhanced luciferase activity in the WT cells, demonstrating that this site indeed mediates the transcriptional activity of p53 in response to UV.

## Discussion

Here we describe a biochemical purification of a DNA damage-responsive p53 Ser-392 kinase complex from mammalian cells. The results from this study demonstrate that we have identified and purified a multisubunit kinase complex that specifically targets Ser-392 and consists of at least seven polypeptides with a native molecular mass of  $\sim$ 700 kDa. Unexpectedly, this complex contains CK2, the FACT heterodimer (hSpt16 and

SSRP1), and two other as yet unidentified proteins. More surprisingly, although it contains CK2, this p53 Ser-392 kinase complex does not appear to target casein, a classical CK2 substrate, suggesting that the other components in this complex may play a role in regulating substrate specificity. The association of CK2 with hSpt16 and SSRP1 was further validated by cofractionation of these proteins and by coimmunoprecipitation with antibodies specifically against hSpt16 and SSRP1. Thus, our study demonstrates that CK2 associates with chromatin-related transcription elongation factor hSpt16 and SSRP1, forming a large protein kinase complex that preferentially phosphorylates Ser-392 of p53. This suggests a possible mechanism that may regulate p53 function in response to DNA damage caused by UV irradiation.

## DNA Damage-Inducible p53 Ser-392 Kinase

Supporting the importance of Ser-392 in p53 regulation, we found that phosphorylation at this site promotes the DNA sequence-specific binding and transactivation ability of this protein in vitro (Figures 7A–7C). Our evidence using a transient transfection system also suggests that Ser-392 is an important site in vivo for regulating p53 activity following UV treatment (Figure 7D). However, because of the limitations of this experimental approach (e.g., overexpressed protein, etc.), it is difficult to analyze the Ser-392 pathway in the context of the multiple pathways that activate p53 following DNA damage. Thus, this question and the role of Ser-392 in cell growth regulation will be further studied by generating a mouse embryonic stem cell line containing a homozygotic S392A mutation, using homologous recombination technology (Chao et al., 2000).

Because Ser-392 is functionally important, identification of the kinase that targets this site is crucial for understanding p53 biology. Two kinases, CK2 and

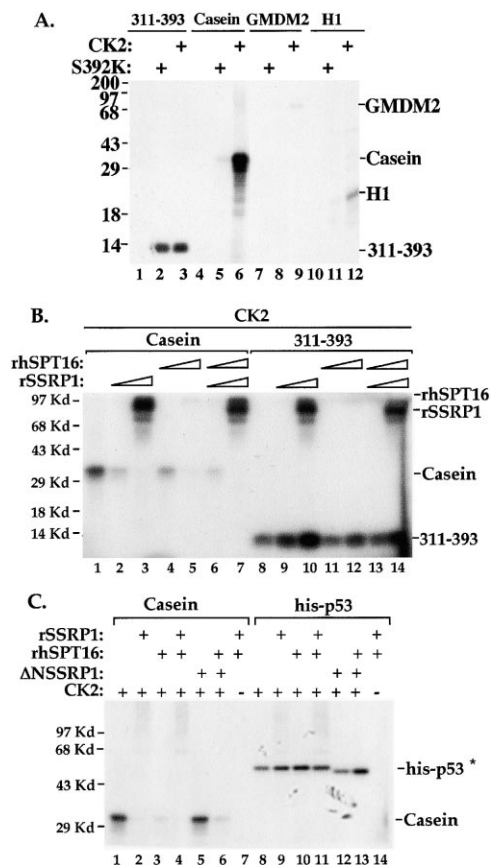


Figure 6. Comparison of the Purified p53 Ser-392 Kinase with CK2 Alone Reveals Different Substrate Specificities

(A) Both the purified p53 Ser-392 kinase complex and CK2 alone phosphorylate C-terminal p53 (aa 311–393, 100 ng) equally well in an in vitro radioactive kinase assay, but p53 Ser-392 kinase does not efficiently phosphorylate casein (1  $\mu$ g), an N-terminal deleted, GST-fusion MDM2 (G $\Delta$ MDM2, 50 ng), or histone H1 (100 ng). CK2 (0.5 U) (Promega) and 300 ng of the purified p53 Ser-392 kinase (pool of fractions 9–11) were used in kinase reactions.

(B) The substrate specificity of the p53 Ser-392 kinase complex is reconstituted in vitro. A radioactive kinase assay was done as above using casein and 311–393, along with his-SSRP1 (4 and 100 ng) and/or Flag-hSpt16 (4 and 100 ng). rhSpt16 and rSSRP indicate recombinant proteins.

(C) The substrate switching as seen above is not due to a nonspecific protein–protein interaction because N-terminal SSRP1 (aa 1–240) does not affect casein phosphorylation. A radioactive kinase assay was done as above with 10 ng  $\Delta$ NSSRP1, 25 ng his-SSRP1, 25 ng Flag-hSpt16, 100 ng his-p53, and 1  $\mu$ g casein. The asterisk denotes the migration of p53 in lanes 12 and 13 changes due to cleavage of the histidine tag by contaminating thrombin in the  $\Delta$ NSSRP1 preparation.

p38MAPK, have been shown to phosphorylate Ser-392 in vitro (Meek et al., 1990; Huang et al., 1999; Keller et al., 1999). p38MAPK was suggested to be responsible for Ser-392 phosphorylation after DNA damage, largely based upon the observations that UV-stimulated Ser-392 phosphorylation was inhibited by p38MAPK-specific inhibitors (Huang et al., 1999; Keller et al., 1999). However, there is no clear evidence demonstrating that this kinase is directly responsible for Ser-392 phosphorylation in response to DNA damage. Although it phos-

phorylates Ser-392 and enhances p53's sequence-specific DNA binding activity in vitro (Hupp et al., 1992), whether CK2 directly regulates p53 functions in vivo in response to DNA damage has remained elusive.

Characterization of the purified p53 Ser-392 kinase from HeLa or F9 nuclear extracts suggests that it is part of a multisubunit complex. Surprisingly, this kinase complex contains CK2. We propose that this may be the major kinase, if not the only one, that specifically targets Ser-392 for the following reasons: (1) Ser-392 presents a well conserved CK2 site (Guerra et al., 1999), but not a p38MAPK site (Obata et al., 2000); and (2) p53 Ser-392 kinase activity was barely detected in any other fractions other than the P11 0.5 M fraction (Figure 2A), from which a kinase complex containing CK2 was purified (Figure 4). p38MAPK may not directly target Ser-392, as this protein was not found in the active fractions, even when samples were prepared from UV-irradiated F9 cells (data not shown). Interestingly, p38MAPK was recently shown to phosphorylate CK2 and activate its kinase activity in cells upon cellular stress (Sayed et al., 2000). This may well explain the inhibition of Ser-392 phosphorylation by p38MAPK-specific inhibitors (Huang et al., 1999; Keller et al., 1999). Therefore, one plausible model for UV-p53 signaling would be that UV activates p38MAPK, which in turn associates with and phosphorylates CK2, and the activated CK2 then targets Ser-392. However, in cells, this simplified mode of p53 activation may be complicated by more complex mechanisms during specific cellular events, as discussed below.

#### The p53 Ser-392 Kinase Complex

We have found that a portion of CK2 in the cell is associated with a high molecular weight complex that specifically targets Ser-392 of p53. When compared with the heterotetrameric CK2 alone, CK2 associated with the protein complex displays different substrate specificity, favoring p53 more than other CK2 substrates, such as casein, MDM2, and histone H1 (Figure 6). Little has been done to show how CK2 is regulated in vivo, despite the fact that it has over 160 known targets (Guerra et al., 1999). Regulation of CK2 may thus be critical for its selective targeting of cellular substrates during a specific cellular event, such as DNA damage, or at a specific time. Our study presents evidence suggesting that CK2 may be regulated by other protein partners that influence its substrate selectivity.

Two proteins associated with CK2 were hSpt16 and SSRP1. These proteins cofractionated with CK2 throughout the purification (Figures 4 and 5A, and data not shown), and we further demonstrated the association by using antibodies against hSpt16 and SSRP1 to immunoprecipitate CK2 $\alpha'$  and p53 Ser-392 kinase activity (Figure 5B). FACT activity was recently identified coincidentally in the P11 0.5 M KCl fraction from HeLa nuclear extracts as a factor that enhanced the processivity of RNA polymerase II through a nucleosome array in vitro (Orphanides et al., 1998), and purified as a heterodimer with the human homologs of *Saccharomyces cerevisiae* Spt16 and yeast Pob3 (SSRP1) (Orphanides et al., 1999). We found that a small portion of hSpt16 and SSRP1 (5%) associates in a high molecular weight complex with CK2 (data not shown). SSRP1 belongs to the HMG box

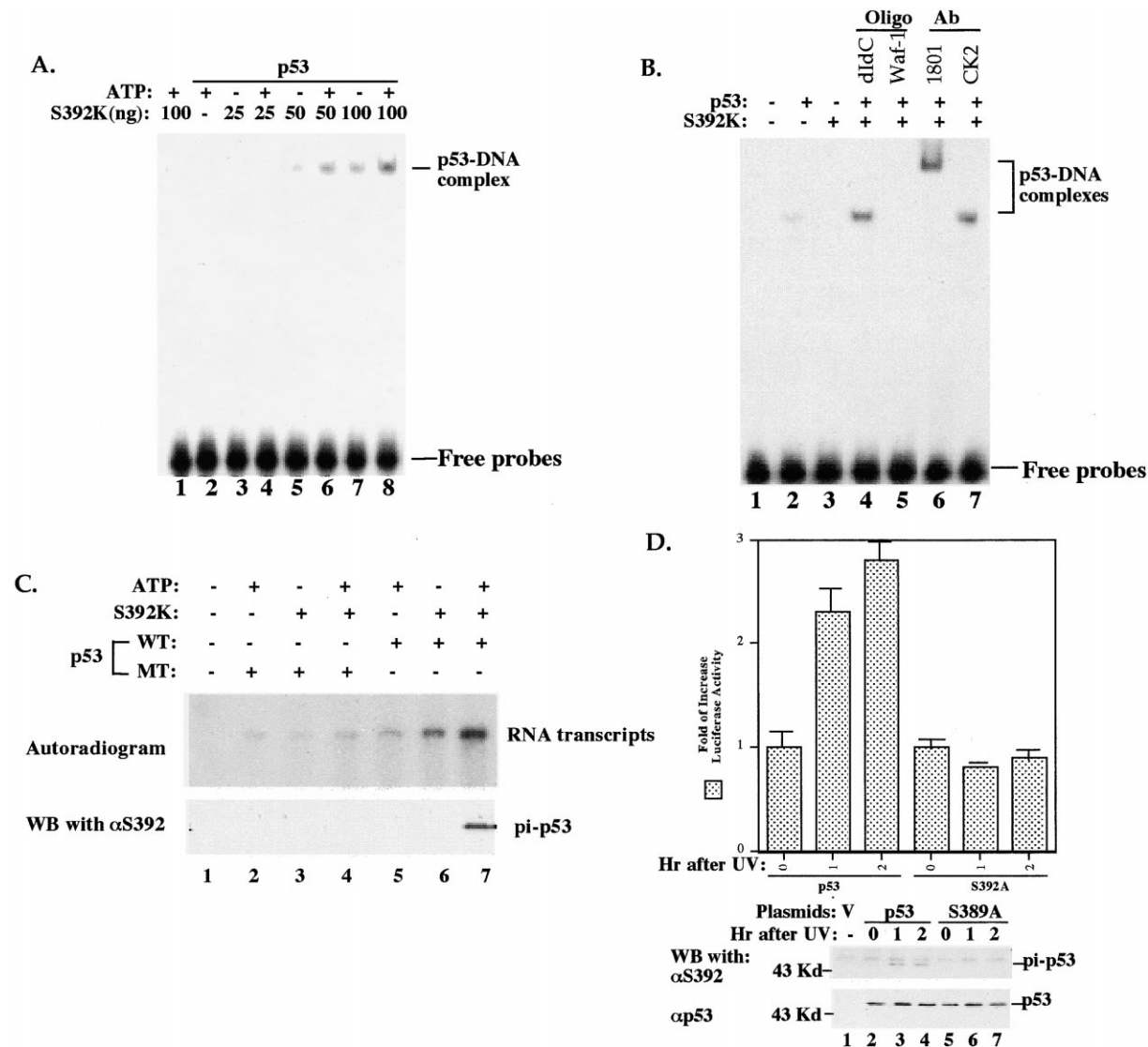


**Figure 7. The Purified p53 Ser-392 Kinase Complex Enhances p53's Sequence-Specific DNA Binding and Transcriptional Activity In Vitro**

(A) Cold kinase assays were performed, followed by an electro-mobility shift assay (EMSA) using full-length his-p53 (50 ng) that was phosphorylated by increasing amounts of purified p53 Ser-392 kinase as indicated. Phosphorylated his-p53 was incubated with a 3' end-labeled DNA probe harboring two copies of the p53RE sequence derived from the p21 waf1/cip1 promoter. S392K indicates the purified p53 Ser-392 kinase.

(B) The DNA/protein complex formed was specific to p53. The same kinase-EMSA assay was conducted as in (A). Two unlabeled oligomers were used, poly(dIdC) (100 ng, lane 4) and p53RE (100 ng, lane 5). Also, antibodies (0.1  $\mu$ g each) were used that were specific against p53 (PAb 1801, lane 6) and CK2 $\alpha'$  subunit (lane 7).

(C) The p53 Ser-392 kinase stimulates transcription mediated by wild-type (WT) but not mutant (MT) p53. A reconstituted transcription system was used containing purified RNA pol II (50 ng), general transcription factors, and a DNA template containing a p53RE motif upstream from the TATA box derived from adenovirus major late promoter. One hundred nanograms of WT or MT p53 and 4  $\mu$ l fraction 12 (S392K, Ser-392 kinase) from Superdex 200 column were preincubated in the presence or absence of ATP prior to being added into the transcription reaction. RNA transcripts were detected by autoradiography (top), and Ser-392 phosphorylation of p53 under the same reaction condition was detected by Western blot using the anti-Ser-392 antibody (bottom).

(D) Ser-392 is critical for p53-dependent transcription in vivo following UV treatment. H1299 cells ( $10^5$ /well, six-well plate) were transfected with plasmids (making up to 1  $\mu$ g DNA in total with a control vector) encoding no gene, murine wild-type p53, or S389A mutant (400 ng), together with a luciferase reporter plasmid (100 ng) driven by the p53RE motif derived from the p21 promoter and a  $\beta$ -galactosidase reporter plasmid (200 ng) driven by the CMV promoter. Forty-eight hours posttransfection, cells were UV-treated for 0, 1, or 2 hr and then harvested for luciferase and  $\beta$ -gal assays. Luciferase activity was normalized by the internal  $\beta$ -gal activity. Fold increase in luciferase activity was calculated and is presented in the graph (each column is from three independent assays, and bars show standard deviation). The bottom panels show p53 expression as detected by immunoprecipitation with PAb 421 and Western blot with either  $\alpha$ Ser-392 or  $\alpha$ p53 polyclonal antibodies.

family of proteins (Bruhn et al., 1992), which are highly abundant non-histone chromatin proteins (Grosschedl et al., 1994). Interestingly, SSRP1 was identified initially

in a peptide library screen for human proteins that bound to DNA modified by the anticancer drug cisplatin (Bruhn et al., 1992). This highlights the fact that SSRP1 has

multiple functions, including DNA damage recognition and transcription elongation.

Identification of the hSpt16-SSRP1-CK2 complex raises the question of how these proteins may regulate p53 activity. As part of the transcription elongation machinery, hSpt16 and SSRP1 are in a prime location for recognizing sites of DNA damage that occur on the transcribed strand. We suggest that CK2 is associated with hSpt16 and SSRP1 during transcriptional elongation and may be the trigger for the kinase complex to phosphorylate p53 when it encounters DNA lesions. This model, although hypothetical, is supported by evidence from the literature. It has been reported that a positive correlation exists between inhibition of mRNA synthesis and activation of p53 (Yamaizumi and Sugano, 1994; Ljungman and Zhang, 1996; Dumaz et al., 1997; Ljungman et al., 1999). RNA polymerase II stalls when it encounters DNA lesions caused by agents such as UV and cisplatin, leading to a drop in mRNA synthesis (Lindahl and Wood, 1999). Accumulation and activation of p53 following UV and cisplatin in normal human fibroblasts was found to closely parallel suppression of mRNA synthesis (Ljungman et al., 1999). The accumulation of p53 appears to be DNA damage dependent because in xeroderma pigmentosum type A (XP-A) cells deficient in nuclear excision repair, p53 was induced at significantly lower levels of UV and cisplatin. Interestingly, and consistent with our proposal, ionizing radiation did not lead to an inhibition of synthesis (Ljungman et al., 1999), which suggests that the p53 Ser-392 kinase complex is activated specifically after UV, but not after  $\gamma$  irradiation.

Several unanswered questions remain, however. Other inhibitors of RNA pol II transcription, both at the stages of initiation and elongation, induce p53 stabilization and activation (Ljungman et al., 1999). Is actinomycin D, which intercalates into the DNA and induces p53 (Kastan et al., 1991), also recognized by SSRP1 and hSpt16? Also, the serine/threonine kinase inhibitor DRB is an effective agent against CK2 (Zandomeni et al., 1986) and can also inhibit transcription initiation (Egyhazi, 1976; Sehgal et al., 1976) and/or elongation (Chodosh et al., 1989). However, DRB does not appear to inhibit CK2 phosphorylation of Ser-392, but rather activates and stabilizes p53 (Blaydes and Hupp, 1998; Ljungman et al., 1999). Perhaps CK2 is not inhibited by DRB when complexed with SSRP1 and hSpt16.

Identification of a p53 Ser-392 kinase complex that contains CK2 and the transcription elongation factor FACT provides a mechanism for how CK2 may regulate p53 in response to DNA damage. Our findings also suggest that CK2 may have a role in transcription or replication regulation. How this complex functions *in vivo* will be an interesting topic of future research.

#### Experimental Procedures

##### Reagents and Buffers

Histone H1 and casein were purchased from Sigma. CK2 was purchased from Promega. Flag-hSpt16 was generously provided by Danny Reinberg (Rutgers, NJ). Buffer C 100 (BC100) contains 20 mM TRIS/HCl (pH 7.9), 0.1 mM EDTA, 15% glycerol, 100 mM KCl, 1 mM DTT, and protease inhibitors including 0.2 mM PMSF, 4  $\mu$ M pepstatin A, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin. BC100 buffer was used for IP assays and included phosphatase inhibitors NaF (100  $\mu$ M) and sodium ortho-vanadate (100  $\mu$ M). Kinase buffer (1 $\times$ ) is 20 mM Tris/HCl (pH 7.5), 10 mM MgCl<sub>2</sub>, and 1 mM DTT.

##### Plasmids and Antibodies

The his-p53 expression vector was obtained from Ze'ev Ronai (Mount Sinai Medical School, New York, NY). pET-311-393 expression vector was generously provided by Nikola Pavletich (Memorial Sloan Kettering Cancer Center, New York, NY). pET-mp53-S392A expression vector was as described (Kapoor and Lozano, 1998). pKK233-3+SSRP1 plasmid was a gift from Stephen Lippard (MIT), and SSRP1 was then subcloned into pET24a expression vector (Novagen). N-terminal SSRP1 (bp 1-719) was generated by PCR and subcloned into pGEX-KG (Pharmacia). Anti-CK2 $\alpha$ ' and polyclonal anti-p53 antibodies were from Santa Cruz Biotech., Inc., and anti-CK2 $\beta$  antibody was from Transduction Laboratory, Inc. Anti-Ser-392 phospho-specific p53 antibody was prepared as previously described (Kapoor and Lozano, 1998), as was PAb421 (Harlow et al., 1981). Polyclonal anti-mSpt16 antiserum was generated specifically against a mouse Spt16 fragment corresponding to human Spt16 aa 802-1047. Polyclonal anti-SSRP1 antiserum was generated against full-length histidine-tagged SSRP1.

##### Cell Culture

Human cervical carcinoma HeLa cells were cultured in suspension in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose and with L-glutamine (GIBCO-BRL), and supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 0.1 mg/ml streptomycin. Small-cell lung carcinoma H1299 cells were cultured on plates with identical media. Murine embryonic testicular carcinoma F9 cells containing wild-type p53 were grown on plates in DMEM without sodium pyruvate, supplemented with 5% FBS and with penicillin/streptomycin. All cells were grown at 37°C in a 5% CO<sub>2</sub> atmosphere.

##### Purification of Recombinant Proteins and Affinity Purification of Antibodies

His-p53, his-SSRP1, and his-mSpt16 (aa 802-1047) were purified on Ni-NTA agarose as per the manufacturer's instructions (Qiagen). N-terminal SSRP1 was bound to glutathione agarose beads (Sigma) and then digested with thrombin protease (Pharmacia) to remove the GST-tag. The C-terminal p53 (aa 311-393) peptide was purified on an HS 4.6/100 column (BioCad Sprint HPLC) and protein was eluted in a 40 ml linear gradient from 0.1 M to 0.8 M KCl (BC-100 to BC-800 buffer). 311-393 peptide eluted at the end of the gradient and was judged  $\sim$ 75% pure by silver staining. Anti-mSpt16 and anti-his-SSRP1 antibodies were purified from antigen affinity columns using CNBr-activated Sepharose 4B (Pharmacia Biotech) as per the manufacturer's instructions, using a protocol from Harlow and Lane (1988).

##### Purification of p53 Ser-392 Kinase

F9 cells were grown to 80% confluency on plates, and then half the plates were treated with 20 J/m<sup>2</sup> UVC ( $2 \times 10^9$  cells, total). Treated cells were harvested 8 hr post-UV, and both treated and untreated cells were used for nuclear extract preparation as previously described (Lu et al., 1998). Nuclear extracts were loaded onto a phosphocellulose (P11) column, and fractions were collected at 0.1 M KCl (BC-100), 0.35 M KCl (BC-350), 0.5 M KCl (BC-500), and 1.0 M KCl (BC-1000). HeLa cells were grown in suspension culture and were used for making nuclear extract ( $10^{10}$  cells). Nuclear extracts were precipitated with ammonium sulfate to 60% saturation. The resuspension was dialyzed overnight against BC-100, and 420 mg protein was loaded onto a P11 column and eluted as described above. The BC-500 fraction (24 mg protein) was loaded onto a DEAE Sephacyl column (Pharmacia Biotech) and step-eluted as above. The BC-350 fraction (14 mg) was further purified on an HQ 4.6/100 column (BioCad Sprint HPLC), and protein was eluted in a 30 ml linear gradient from BC-100 to BC-800. Active fractions (4.1 mg) were pooled and run on an HS 4.6/100 column and eluted in the same manner. Active fractions (1.2 mg) were again pooled and loaded onto a Heparin Hi-Trap column (1 ml, Pharmacia). A 20 ml linear gradient was run, again from BC-100 to BC-800, and active fractions (500  $\mu$ g) were pooled. The protein was then concentrated on a MonoQ (1.6/5) column (Smart HPLC, Pharmacia), and active fractions were further concentrated with a Microcon microconcentrator spin column (Amersham). Fifty milliliters of sample was run on a Superdex 200 (3.2/30) column (Smart HPLC, Pharmacia). p53 Ser-392 kinase

activity eluted with a native molecular weight of 700 kDa as judged by molecular weight standards (BioRad).

#### Identification of Protein Sequences

Polypeptides were digested in gel and analyzed by using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and electrospray ion trap mass spectrometry (ES-ITMS) in combination with capillary HPLC. As a result, 15 peptides of p80 matched with 100% of the calculated tryptic peptide masses from SSRP1, covering 23% of the protein sequence. Electrospray tandem mass spectrometry coupled with liquid chromatography (LCQ/MS/MS) analysis of p140 revealed a peptide mass 100% matching an EST peptide (VDNIQAGELTEGIWRFDEISFVNFR). This peptide perfectly matches the aa 577–601 region of hSpt16. Analysis of p41, p38, and p27 showed several peptides matching the regions of CK2 subunits (p41, GSRARVYAEVNSLRSEYRW; p37, VLGTEELYGYLKK; p27, FNLTGLNEQVPHYR).

#### In Vitro p53 Ser-392 Kinase Assay

The p53 Ser-392 kinase assay was carried out using a previously described method (Lu et al., 1997) using [ $\gamma$ - $^{32}$ P]-ATP. Substrates included 100 ng of his-p53, 100 ng of 311–393, 100 ng histone H1, 200 ng G $\Delta$ MDM2, and 1  $\mu$ g casein. Either 3  $\mu$ l of the column fractions or 20 ng of the purified p53 Ser-392 kinase or 0.5 U CK2 (Promega) was used. Kinase assays were also done using unlabeled ATP (1 mM) followed by SDS-PAGE, and then phosphorylated 311–393 was detected by Western blot using the anti-Ser-392 antibody.

#### Western Blotting, Immunoprecipitation, and IP-Kinase Assays

Protein samples were run on SDS-PAGE, followed by transfer to nitrocellulose membranes that were then immunoblotted with the appropriate antibodies. Proteins were detected by ECL reagents (Santa Cruz Biotech). hSpt16, SSRP1, and CK2 $\alpha'$  were immunoprecipitated from the P11 0.5 M KCl fraction. Anti-hSpt16 or anti-hSSRP antibodies (1  $\mu$ g) were combined with 10  $\mu$ l protein G-Sepharose 4B fast-flow beads (Pharmacia) and 50  $\mu$ g protein for 2 hr at 4°C in a total volume of 400  $\mu$ l BC100 buffer. The IPs were washed three times in BC100 and one time in 1 $\times$  kinase buffer, and then immediately used in a kinase reaction. A cold kinase assay using unlabeled ATP was then performed as described above using the 311–393 substrate.

#### Kinase/Electro-Mobility Shift Assay, In Vitro Transcription Assay, and Luciferase Assay

Kinase assays were performed as described above, except that reaction volumes were 10  $\mu$ l. Following this, a DNA binding assay was performed as previously published (Kern et al., 1991; Lu et al., 1997). The protein components as shown in the figure legends were incubated with a 3' end-labeled DNA fragment harboring two copies of the p53 response-element (p53RE) sequence (5000 cpm, 0.1–1.0 ng DNA/assay) derived from the p21waf1/cip1 promoter (Zeng et al., 1998) for 20 min at room temperature in a final volume of 20  $\mu$ l. Unlabeled oligomers (100 ng), including poly(dIdC) and p53RE, were used for competition studies. Antibodies (0.1  $\mu$ g), including the p53 antibody Pab 1801 and anti-CK2 $\alpha'$  antibody, were used to test the specificity of the DNA/protein complex. The DNA/protein complexes were separated by electrophoresis through a 5% native acrylamide gel containing 4% glycerol and detected by autoradiography. The transcription assay was done as described (Lu et al., 1992; Lu and Levine, 1995). The transient transfection and luciferase assays were done as described (Zeng et al., 2000).

#### Acknowledgments

We thank Kathleen M. Alexander for helping in preparation of this manuscript and Stephen Lipard, Danny Reinberg, Nikola Pavletich, and Ze'ev Ronai for generously providing us with reagents used in this study. D. M. K. is supported by the NEI predoctoral fellowship. Q. H. Z. and R. G. are supported by NIH grants. Y. W., H. S., and

Y. Z. are supported by NIH grants. This work is partly supported by grants to H. L. from NIH and ACS.

Received July 7, 2000; revised December 26, 2000.

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