

p53 Serine 392 Phosphorylation Increases after UV through Induction of the Assembly of the CK2-hSPT16-SSRP1 Complex*

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Previously, we purified a UV-responsive p53 serine 392 kinase from F9 and HeLa cells and found that its activity is attributed to a high molecular weight protein complex containing the protein kinase CK2, along with the chromatin-associated factors hSPT16 and SSRP1. Here we determine that these proteins interact *in vitro* and in cells via non-overlapping domains and provide evidence consistent with the idea that hSPT16 and SSRP1 change the conformation of CK2 upon binding such that it specifically targets p53 over other substrates. Also, UV irradiation apparently induces the association of the complex, thereby increasing the specificity of CK2 for p53 at the expense of other cellular CK2 substrates and leading to an overall increase in p53 serine 392 phosphorylation.

The tumor suppressor protein p53 is a highly connected cellular sensor of DNA damage and aberrant cell growth and serves a protective role by inhibiting the cell cycle or inducing apoptosis once damage occurs (1). Cellular insults that activate p53 include DNA-damaging agents such as radiation and chemical mutagens (2–5), hypoxia (6), ribonucleotide depletion (7), oxygen free radicals (8), and cellular and viral oncogenes (9, 10). After such stresses, p53 is converted from a latent form into an active transcription factor and primarily induces the expression of numerous target genes involved in cell damage control (1, 11). Concurrently, p53 protein levels dramatically increase through relief of targeted degradation to the proteasome (12, 13) and through increased translation of the p53 mRNA (14–16). Thus many stress-activated signaling pathways connect to p53, which in turn activate expression of downstream effector pathways (1).

One of the mechanisms by which stress signals are communicated to p53 is through post-translational modifications (17). These modifications such as phosphorylation and acetylation are believed to control the protein stability of p53 and transcriptional activity by affecting protein-protein interactions and intramolecular conformational changes (11). For example, phosphorylation at several sites on the N-terminal domain in response to stress (5, 18–21) prevents binding of the MDM2 oncoprotein, an E3¹ ubiquitin ligase that targets p53 for deg-

radation by the proteasome pathway (22–24). On the C terminus, phosphorylation of Ser-392 (corresponding to murine Ser-389, for simplicity, Ser-392 will be used) enhances DNA sequence-specific binding and transcription activity *in vitro* (25, 26), possibly by stabilizing p53 tetramerization (27). In cells, the importance of Ser-392 phosphorylation for p53 function appears to be situation-specific. For example, overexpression of a p53 Ser-392 > Ala mutant suppressed cell growth equal to wild-type p53 in human osteosarcoma SAOS2 cells, but instead impaired the ability of p53 to suppress ras-mediated transformation in rat embryonic fibroblasts (28). Also, transient transfection of p53 with a Ser-392 > Glu substitution, but not with six other phosphorylation mutants, constitutively activated p53 as a transcription factor in NIH 3T3 mouse fibroblasts after cell growth arrest by contact inhibition (29). Taken together, these results suggest that p53 Ser-392 is important for full p53 function.

In multiple cell types, p53 Ser-392 is phosphorylated specifically after UV but not γ irradiation or etoposide (3, 4). Casein kinase 2 (CK2) was originally identified as the kinase that targets this site *in vitro* (30). However, it was unclear what the true kinase that targets this site is in cells. Previously, we used a biochemical fractionation to purify UV-responsive p53 Ser-392 kinase activity from murine testicular carcinoma F9 cells (26). Our results showed that indeed the kinase was CK2, but it eluted from gel filtration chromatography in a high molecular weight fraction corresponding to ~700 kDa. We identified two other proteins that eluted with CK2 as hSPT16 and SSRP1. Together, these molecules are known in mammals as the chromatin associated factor, FACT (31). Interestingly, when hSPT16 and SSRP1 are complexed with CK2, they change the substrate specificity of CK2 to phosphorylate p53 over all other tested substrates. However, it remains unclear how CK2 preferentially targets p53 as a substrate after association with hSPT16 and SSRP1 and how this kinase complex is activated by DNA damaging signals.

To determine the mechanism by which the p53 Ser-392 kinase complex is activated by DNA damage, we have further characterized this complex *in vitro* and in cells. First, we have mapped the interaction domains between CK2, hSPT16, and SSRP1 *in vitro* and in cells, demonstrating that these proteins interact with each other via non-overlapping regions, consistent with the idea that they form a complex. Second, steady-state kinetic analysis of the kinase activity of CK2 shows that

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¹ The abbreviations used are: E3, ubiquitin-protein ligase; CHD1,

chromodomain-helicase-DNA binding; CK2, casein kinase 2; DTT, dithiothreitol; FACT, facilitates chromatin transcription; GST, glutathione S-transferase; SSRP1, structure-specific recognition protein; hSPT16, human ortholog of yeast suppressor of Ty insertion mutations; IP, immunoprecipitation; MDM2, a protein encoded by a gene amplified in mouse double-minute chromosome; P11, phosphocellulose; WB, Western blot; WT, wild-type; aa, amino acid(s).

binding of hSPT16 and SSRP1 to CK2 inhibits casein phosphorylation while having no effect upon p53 phosphorylation and that hSPT16 and SSRP1 apparently do not bind to the substrate binding pocket of CK2 but instead are inhibiting casein phosphorylation in an indirect fashion. Furthermore, we find that hSPT16, SSRP1, and CK2 protein levels are dramatically increased in the column fraction that contains the UV-responsive p53 Ser-392 kinase activity. Thus these results demonstrate that the association of CK2 with SSRP1 and hSPT16 in cells is induced by DNA damage signals leading to specific targeting of p53 at Ser-392.

EXPERIMENTAL PROCEDURES

Reagents and Buffers—Casein was purchased from Sigma. CK2 was purchased from Promega. Baculovirus expressing FLAG-WT-hSPT16 was as described (26). 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 phenylmethylsulfonyl fluoride, 4 μ M pepstatin A, 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin. BC100 buffer was used for IP assays and included phosphatase inhibitors NaF (100 μ M) and sodium orthovanadate (100 μ M). Kinase buffer (1 $\times$) is 20 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, and 1 mM DTT. Lysis buffer consists of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, and protease inhibitors as above. Radioimmune precipitation assay buffer is 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM DTT, and protease inhibitors as above.

Plasmids and Antibodies—The His-p53 and pET-311-393 expression vectors were as described previously (26). The pRc/CMV/CK2 α' -HA and pRc/CMV/CK2 β -myc were generous gifts from David Litchfield (32), and the CK2-encoding cDNAs were subcloned into pET24a expression vectors (Novagen). pET28-antisense hSPT16 was from Danny Reinberg (University of Medicine and Dentistry in New Jersey, Robert Wood Johnson Medical School, NJ) and was used in PCR to generate N-terminal (aa 1–329) and mid-hSPT16 (aa 321–640) followed by subcloning into pET24a and pGEX-KG (Amersham Biosciences) expression vectors. pKK233-3+SSRP1 plasmid was as described previously (26) and SSRP1 was then subcloned into pET24a and pGEX-KG expression vectors. N-terminal SSRP1 (aa 1–242), mid-SSRP1 (aa 235–475), and C-SSRP1 (aa 471–709) were generated by PCR and subcloned into pGEX-KG. N- and C-SSRP1 were also subcloned into a FLAG-modified pCDNA3.1 mammalian expression vector (Invitrogen). Polyclonal anti-CK2 α' and anti-p53 antibodies were from Santa Cruz Biotechnology, Inc, monoclonal anti-CK2 β antibody was from Transduction Laboratories, Inc, and monoclonal anti-FLAG and anti- γ -tubulin antibodies were from Sigma. Anti-Ser-392 phosphospecific p53 antibody was prepared as previously described (3, 18) as was PAb421 (33). Polyclonal anti-SSRP1 antiserum was generated against full-length histidine-tagged SSRP1 and polyclonal anti-hSPT16 antiserum was made against the middle portion of the protein (aa 321–640).

Purification of Recombinant Proteins and Affinity Purification of Antibodies—Histidine-tagged proteins were purified on nickel-nitrilotriacetic acid agarose as per the manufacturer's instructions (Qiagen). GST fusion proteins were bound to glutathione-agarose beads (Sigma) and then were either left on the beads or were digested with thrombin protease (Amersham Biosciences) to remove the GST tag. The anti-SSRP1 antibody was purified as described previously (26).

Cell Culture—Murine embryonic testicular carcinoma F9 cells containing wild-type p53 were grown on plates in Dulbecco's modified Eagle medium with 4.5g/liter glucose and with L-glutamine (Invitrogen), supplemented with 5% fetal bovine serum and penicillin/streptomycin. Human colorectal carcinoma RKO cells were grown on plates in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. All cells were grown at 37 °C in a 5% CO₂ atmosphere.

GST Fusion Protein Association Assay—GST fusion proteins overexpressed in bacteria were purified on glutathione-agarose beads (Sigma) as described by the manufacturer. The GST fusion protein levels were then equalized by loading onto SDS-PAGE and visualized with Coomassie Brilliant Blue. 1 μ g of fusion proteins were combined with 1 μ g of soluble His-WT-SSRP1, FLAG-WT-hSPT16, His-CK2 α' , or His-CK2 β and incubated at room temperature for 40 min with light vortexing. The samples were washed once with lysis buffer, once with 1:3 diluted lysis buffer in water, once with radioimmune precipitation assay buffer, and once with lysis buffer. They were then run on SDS-PAGE and transferred to a polyvinylidene difluoride membrane for Western blotting (WB). GST-pull-down kinase assays were performed as above except

that GST fusion proteins were combined with 1 unit of CK2 followed by washing three times with lysis buffer and once with 1 $\times$ kinase buffer. WB-kinase reactions were carried out as previously described (26, 34) using ATP and 100 ng of His-p53 as substrates for 30 min at 30 °C.

Generation of FLAG-N- and C-SSRP1 Cell Lines—RKO cells were transfected with 3 μ g of either pCDNA3.1, pCDNA3.1 FLAG-N-SSRP1, or pCDNA-FLAG-C-SSRP1 expression constructs using Lipofect-AMINE reagent (Invitrogen). 24 h post-transfection, cells were trypsinized and transferred to 10-cm plates at low density. 0.5 mg/ml G418 was added to the media as a selectable marker, and the cells were maintained for 2–3 weeks until colonies became visible. Individual colonies were expanded into 12-well plates and screened for FLAG-N- or C-SSRP1 protein expression by WB with anti-FLAG and anti-SSRP1 antibodies.

Western Blotting (WB), Co-immunoprecipitation (co-IP), IP Kinase Assays, and In Vitro Kinase Assays—WB, co-IP, and IP kinase assays were carried out as previously described (26). The WB in Fig. 6B was analyzed by a Bio-Rad Model GS700 imaging densitometer. FLAG-N- and FLAG-C-SSRP1 RKO cell lysates were used to perform co-IPs with the anti-FLAG antibody. hSPT16, SSRP1, and CK2 α' were immunoprecipitated with anti-SSRP1 from the P11 0.5 M KCl fractions of the F9 cell nuclear extract preparations as previously described. Radioactive *in vitro* kinase assays were performed with [γ -³²P]ATP, in which the total ATP concentration (cold and hot) was 40 μ M. Substrates were either 100 ng of His-p53 or 1 μ g of casein. In Fig. 4C, WT-SSRP1, N-hSPT16, and mid-hSPT16 proteins are histidine-tagged. N-SSRP1, mid-SSRP1, and C-SSRP1 are thrombin-cleaved from GST while bound to the glutathione-agarose. WT-hSPT16 is FLAG-tagged. Alternatively, kinase assays were done using unlabeled ATP (1 mM) followed by SDS-PAGE and then phosphorylated His-p53 was detected by WB using the anti-Ser-392 antibody.

Kinetic Analysis—CK2 (0.5 unit) was incubated with or without 4 pmol of FACT (2 pmol of FLAG-hSPT16 and 2 pmol of His-SSRP1) on ice for 1 h. *In vitro* radioactive kinase assays were then carried out in the presence of 250 μ M ATP (including 375 μ Ci of [γ -³²P]ATP) for 0.5 h while titrating substrates casein and His-p53. Casein concentrations ranging from 0.25 to 64 μ M and His-p53 ranging from 0.037 to 9.4 μ M were titrated into the CK2-FACT-ATP mixture. Reactions were analyzed by SDS-PAGE, the bands were cut out, and radioactivity was quantified using a Beckman model LS 6500 scintillation counter. Reaction velocities were obtained by measuring the picomoles of ATP incorporated into substrate per second and plotted against substrate concentration. Data points were then fit to the Michaelis-Menten equation using Kaleidagraph (Synergy Software) to obtain values of V_{max} , K_m , and V/K .

RESULTS

The Recombinant FACT-CK2 Complex Has the Same Apparent Molecular Weight as the Native Complex—In our previous study, the purified p53 Ser-392 kinase complex from murine testicular carcinoma F9 cells eluted from gel filtration chromatography at ~700 kDa (26). Seven polypeptides co-eluted with the kinase activity, five of which were identified as SSRP1, hSPT16, or the subunits of the CK2 heterotetramer. To determine whether hSPT16, SSRP1, and CK2 together can form a complex *in vitro*, we incubated these recombinant proteins together and loaded them onto a Superdex 200 size exclusion column (Fig. 1). When CK2 was run on the column alone, it eluted at close to the predicted molecular mass of the heterotetramer, 140 kDa (*top panel*). Interestingly, when recombinant hSPT16 and SSRP1 were mixed with CK2 and then run on the column, the p53 kinase activity of CK2 shifted to a high molecular mass fraction, at ~670 kDa, and at a similar molecular weight to the native p53 Ser-392 kinase complex (*middle panel*, and see Ref. 26). The *bottom panel* is a Western blot for hSPT16 and SSRP1 when combined with CK2. The combined molecular masses of the proteins in the FACT-CK2 complex is predicted to be 360 kDa; thus we speculate that the recombinant complex contains multiple copies of some or all the components. Also, hSPT16, SSRP1, and CK2 appear to be the primary components of our previously purified native kinase complex, although it is still likely that other proteins may associate with this complex in cells.

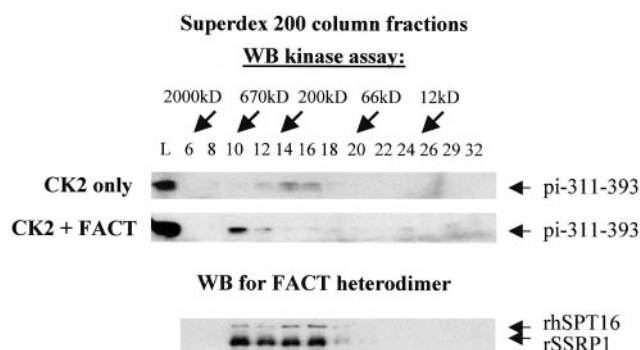


FIG. 1. **The recombinant CK2-FACT complex has the same apparent molecular weight as the purified native one.** CK2 (Pro-mega, 0.7 pmol) was run on a Superdex 200 column, and fractions were analyzed in an α Ser-392 WB kinase assay as described under "Experimental Procedures" (top panel). Next, recombinant FLAG-hSPT16 (rh-SPT16, 20 pmol) and His-SSRP1 (rSSRP1, 20 pmol) were incubated with CK2 on ice for 1 h followed by running on Superdex 200 and analysis by kinase assay (middle panel). The FACT heterodimer with CK2 elutes in fractions 10–16 as visualized by WB in the bottom panel. Ser-392 kinase activity is shifted from fraction 14 without FACT to fraction 10 with FACT, demonstrating a direct interaction *in vitro*.

Mapping of the Protein-Protein Interaction Domains of the SSRP1-hSPT16-CK2 Complex—To characterize the interactions between members of the p53 Ser-392 kinase complex, we performed *in vitro* glutathione *S*-transferase (GST) binding assays. GST-SSRP1 fusion proteins were made with either WT SSRP1 or with three deletion mutants that spanned the length of the protein (Fig. 2A). We generated mutants for both the N terminus and middle region of hSPT16 (Fig. 2A) but were unable to generate either the WT or the C-terminal hSPT16 fusion proteins, because the C terminus of hSPT16 is apparently toxic to bacteria (our observations).² The Coomassie-stained SDS-PAGE gel in Fig. 2A shows that equal levels of the proteins were used, including a GST only (GST-0) control.

As shown in Fig. 2B, FLAG-WT-hSPT16 generated in baculovirus binds to GST-WT-SSRP1 *in vitro* and also to GST-N-SSRP1 (amino acids 1–242), although with an apparent decrease in affinity (left panel, compare lanes 7 and 8). In the reverse experiment, His-WT-SSRP1 binds to GST-mid-hSPT16 (aa 321–640) (Fig. 2B, right panel, lane 6). CK2 binding to SSRP1 and hSPT16 was tested by GST-pull-down followed by a kinase assay. Fig. 2C shows that CK2 binds to GST-WT-SSRP1 and to GST-C-SSRP1 (aa 471–709) (Fig. 2C, left panel, compare lanes 2 and 5) and that there is also diminished binding to GST-mid-SSRP1 (aa 235–475) (compare lanes 2 and 4). CK2 also binds hSPT16 directly *in vitro* via the N terminus (aa 1–329) (Fig. 2C, right panel, lane 2). Thus, from these experiments we conclude that these proteins bind to each other via non-overlapping regions, consistent with the idea that they form an SSRP1-hSPT16-CK2 protein complex. The data are summarized in Fig. 2D.

Protein kinase CK2 exists as a heterotetramer with catalytic subunits α and α' and regulatory subunit β , the stoichiometry being $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ (35). To determine which of these subunits bind to SSRP1 and hSPT16, we used either GST-WT-SSRP1 or GST-N-hSPT16, the region that interacts with the CK2 heterotetramer (Fig. 2C). We tested the catalytic α' subunit and found that it binds to both GST-WT-SSRP1 and GST-N-hSPT16 (Fig. 3A, compare lanes 5 and 6), whereas the regulatory β subunit apparently binds much more strongly to GST-WT-SSRP1 (Fig. 3A, compare lanes 8 and 9). A Coomassie Blue-stained gel is shown of the purified His-tagged CK2 proteins (Fig. 3B).

To test whether these protein-protein interaction domains are also true in cells, we made stable cell lines with FLAG-tagged N- and C-SSRP1 in human colorectal carcinoma RKO cells and performed co-immunoprecipitations (co-IPs) with the FLAG antibody. Using these cell lines, we reproduced the results seen in the *in vitro* GST pull-down assay exactly. That is, FLAG-N-SSRP1 bound exclusively to endogenous hSPT16 (Fig. 3C, top panel, lane 2) and FLAG-C-SSRP1 bound exclusively to the endogenous CK2 α' subunit and p53 Ser-392 kinase activity (Fig. 3C, bottom two panels, lane 3). Therefore, these cellular data confirm the SSRP1 interactions with hSPT16 and CK2 that were observed in the *in vitro* GST pull-down assay. Interestingly, although recombinant GST-C-SSRP1 migrates on SDS-PAGE faster than GST-N-SSRP1 (Fig. 2A, compare lanes c and e), FLAG-C-SSRP1 stably expressed in RKO cells migrates slower than FLAG-N-SSRP1 (Fig. 3C, compare lanes 2 and 3). One possibility for the slower migration of FLAG-C-SSRP1 is that the C terminus of SSRP1 is highly modified in cells by post-translational modifications; this is supported by the fact that this region has a high serine content and that this region is phosphorylated *in vitro* by CK2 (see Fig. 4).

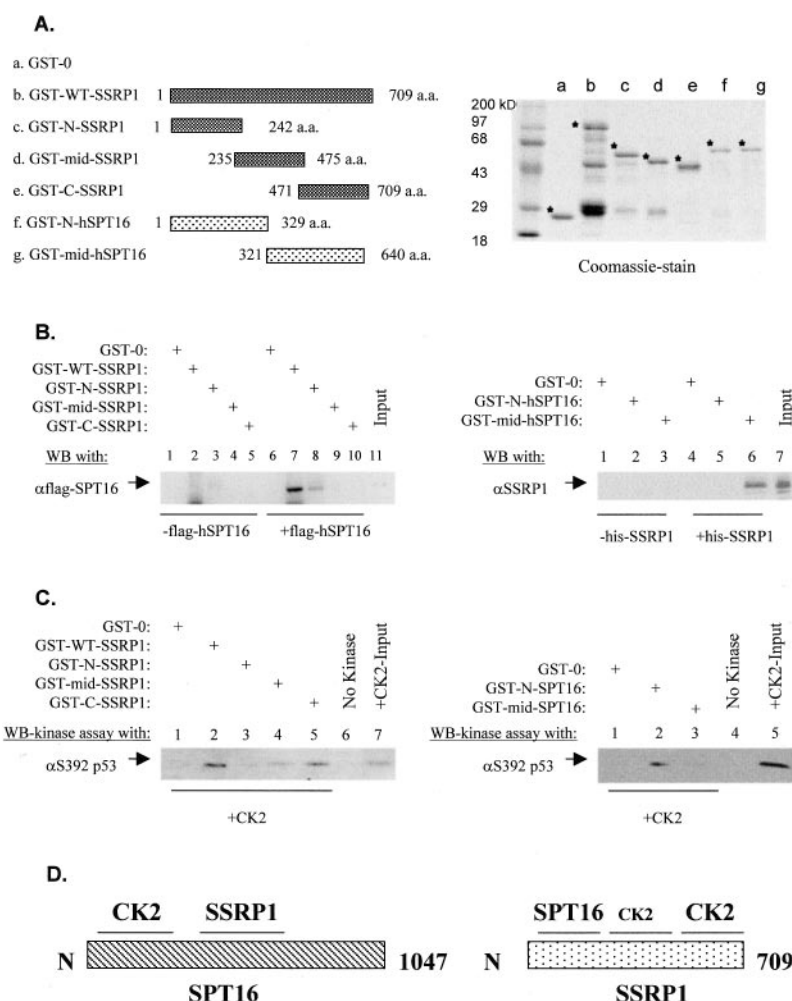
Based upon our protein-protein interaction experiments, we can present a model for the binding of hSPT16 and SSRP1 to the CK2 heterotetramer (Fig. 3D). The CK2 crystal structure is solved (36) and resembles a butterfly, with the two regulatory β subunits making contacts along a 2-fold axis of symmetry, and the catalytic α and α' subunits situated like the butterfly wings making contacts only with one β subunit. Because the molecular weights of the recombinant and native complexes are similar, we speculate that there are two FACT heterodimers bound per CK2 heterotetramer, which would be a predicted size of 580 kDa, close to the 670 kDa estimated size from gel filtration chromatography.

Effect of hSPT16 and SSRP1 Deletion Mutants on CK2 Activity—Previously, we discovered that SSRP1 and hSPT16 could modulate the kinase activity of CK2 such that it phosphorylated p53 but inhibited its activity toward other substrates such as casein, histone H1, and MDM2 (26). Here we identify the p53 family member p63 γ as an *in vitro* substrate for CK2 (Fig. 4A, lane 4), although p63 γ and p53 do not share sequence conservation in the C-terminal domain and there is no p63 γ equivalent of Ser-392. We also tested p73 α , another p53 family member, but found that CK2 does not phosphorylate this protein (Fig. 4A, lanes 7–9). Surprisingly, hSPT16 and SSRP1 inhibit the CK2-induced phosphorylation of p63 γ (compare lane 4 with lanes 5 and 6), providing more evidence for the specificity of the p53 Ser-392 kinase complex.

As described above, we identified the regions of SSRP1 and hSPT16 that directly bind to CK2 and could now test whether these truncation mutants were sufficient to modulate the kinase activity of CK2 also. Kinase assays were performed using either p53 or casein as substrates, with the addition of the various WT and mutant SSRP1 and hSPT16 proteins. As shown in the bottom panel of Fig. 4C, and as seen previously (26), casein phosphorylation was strongly inhibited by His-WT-SSRP1 (compare lanes 1 and 2), although p53 phosphorylation was not affected (Fig. 4, top panel, compare lanes 1 and 2). N-SSRP1, which does not bind CK2, also did not affect CK2 activity toward casein or p53 (compare lanes 3 and 4). In contrast, both mid-SSRP1 and C-SSRP1 inhibited casein phosphorylation (compare lanes 5–8), although mid-SSRP1 inhibited to a greater degree. However, this result was surprising, because C-terminal SSRP1 bound more strongly to CK2 (Fig. 2C). As seen previously, casein phosphorylation by CK2 was inhibited by FLAG-WT-SPT16 (Fig. 4C, bottom panel, lane 9),

² D. Reinberg, personal communication.

FIG. 2. Mapping the hSPT16-SSRP1-CK2 interacting domains. *A*, schematic showing GST fusion proteins (*left panel*). GST-0, GST-SSRP1, and GST-hSPT16 fusion proteins were run on SDS-PAGE and stained with Coomassie Brilliant Blue (*right panel*). The asterisks indicate GST fusion proteins. *B*, FLAG-WT-hSPT16 binds to the N terminus of SSRP1, and His-WT-SSRP1 binds to the middle region of hSPT16 *in vitro*. GST-0 and GST-SSRP1 fusion proteins (1 μ g), immobilized on glutathione-agarose, were incubated either with 1 μ g of FLAG-WT-hSPT16 or without for 40 min at room temperature (*left panel*). Binding reactions were run on SDS-PAGE, and hSPT16 was visualized by WB. In a similar fashion, GST-hSPT16 fusion proteins (1 μ g) were incubated with or without 1 μ g of His-WT-SSRP1 (*right panel*). *C*, CK2 binds to the middle domain and C terminus of SSRP1 and binds to the N terminus of hSPT16 *in vitro*. The indicated GST fusion proteins were incubated with the CK2 heterotetramer (1 unit) and used in a WB kinase assay with 100 ng of His-p53 as a substrate. *D*, schematic of the CK2-hSPT16-SSRP1 protein-protein interactions, based on the above GST-pull-down experiments.



although the truncation mutants appeared to inhibit only slightly (*lanes 10–13*). These data provide evidence that these defined protein-protein interactions of SSRP1 and hSPT16 with CK2 confer substrate specificity upon the kinase.

We also have previously observed that SSRP1 is strongly phosphorylated by CK2 (26). Our current data show that there are at least two phosphorylation sites for CK2 on SSRP1, one covered by the central domain truncation mutant and the other covered by the C-terminal truncation (Fig. 4C, *top panel*, *lanes 4–8*). Although these data will be a subject of future research, it is intriguing to speculate that phosphorylation of SSRP1 may regulate its function as a chromatin-associated transcription and replication factor (31, 37, 38) or in its ability to bind to damaged DNA (39).

Kinetic Analysis of the Phosphorylation Reaction by the SSRP1-hSPT16-CK2 Kinase Complex—To gain insight into how SSRP1 and hSPT16 influence CK2 substrate specificity, steady-state kinetic analysis of CK2 was performed. *In vitro* kinase assays were performed using CK2 with or without SSRP1 and hSPT16 (together known as FACT) and using casein or p53 as substrates. Incorporation of [γ - ^{32} P]ATP into the substrates was measured per second and plotted *versus* substrate concentration and the data was fitted to the Michaelis-Menten equation (Fig. 5) (see “Experimental Procedures” for details). The kinetic parameters show that, when casein is used as the substrate, FACT induces a 2-fold decrease in the maximal reaction velocity (V_{max}) and a 7- to 8-fold increase in the

Michaelis-Menten constant (K_m), a measure of the efficiency of substrate utilization (Fig. 5A). The efficiency of the enzymatic reaction (V/K) is also severely affected (~ 15 -fold decrease) (Fig. 5A). In contrast, p53 phosphorylation by CK2 is not affected by addition of FACT (Fig. 5B), clearly demonstrating that FACT selectively modulates the kinase activity of CK2 by inhibiting its activity against other substrates. These data therefore suggest that the conformation of CK2 is modulated by FACT such that it preferentially targets p53.

The Level of the hSPT16-SSRP1-CK2 Complex Increases after UV—p53 Ser-392 phosphorylation occurs after UV but not γ irradiation in many cell types (3, 4). In our original purification of the p53 Ser-392 kinase, we observed UV-responsive kinase activity in the phosphocellulose (P11) 0.5 M KCl fraction and used this fraction to purify the SSRP1-hSPT16-CK2 complex (Fig. 6A) (26). To understand how the P11 0.5 M KCl fraction had higher p53 Ser-392 kinase activity after UV, we repeated the F9 nuclear extract fractionation and performed WB analysis on the column input and fractions using antibodies against hSPT16, SSRP1, and CK2 α' . Surprisingly, when both non-treated and UV-treated F9 cell nuclear extracts were run on the P11 column and fractionated, hSPT16, SSRP1, and CK2 α' protein levels all were dramatically increased in the UV-treated 0.5 M KCl fraction (Fig. 6B, *top two panels*, *lanes 3 and 4*). This increase was not due to unequal loading, as demonstrated by equal levels of γ -tubulin (*third panel from top*), nor was it due to an overall induction of these proteins after UV,

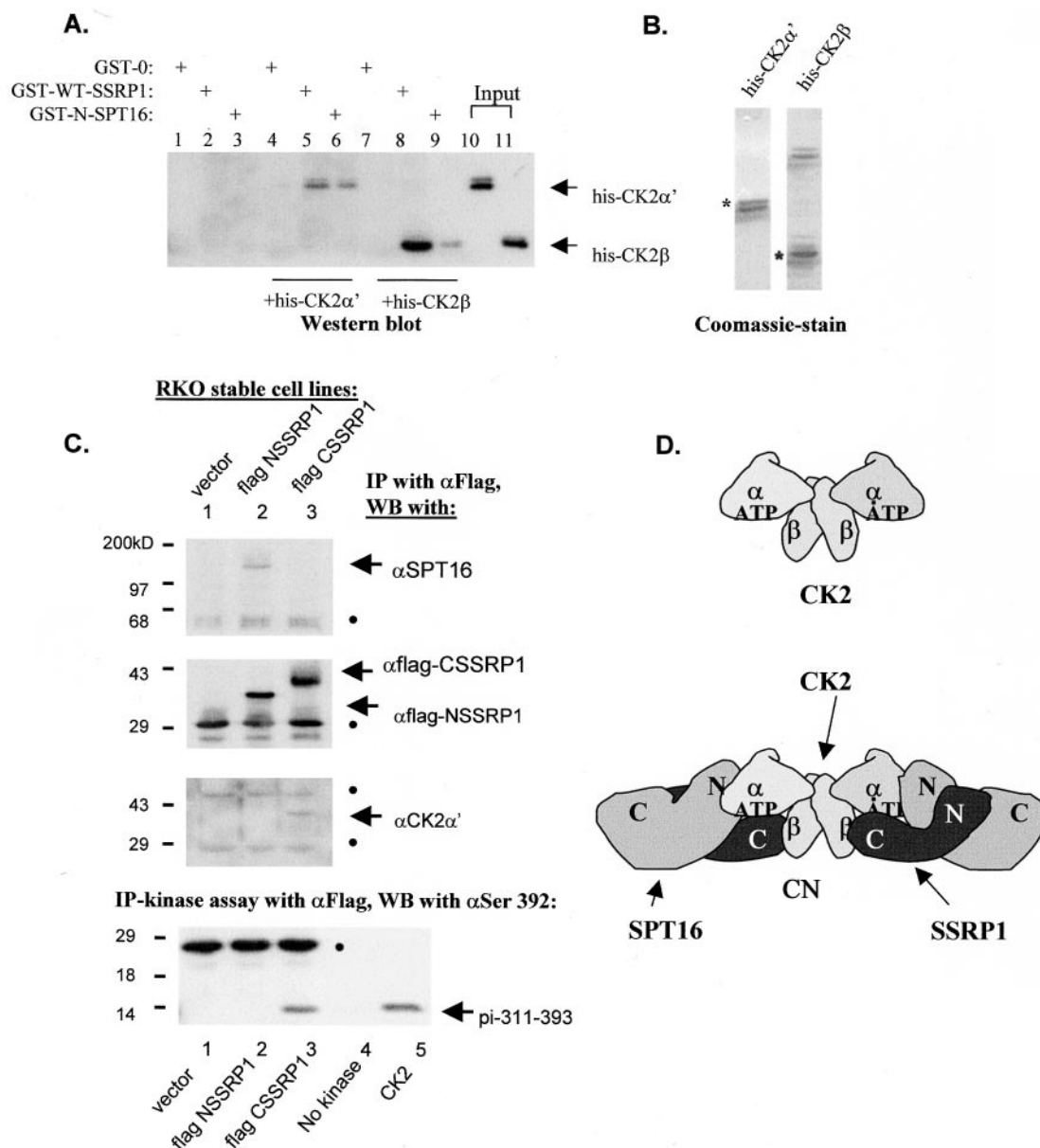


FIG. 3. Characterization of the hSPT16-SSRP1-CK2 complex. A, mapping the CK2 subunit interaction with hSPT16 and SSRP1. CK2 α' binds to both SSRP1 and hSPT16, whereas CK2 β apparently binds more strongly to SSRP1. GST fusion protein binding assays were done as above using 1 μ g of either CK2 α' or β . 10% of the input was loaded directly onto the gel (lanes 10 and 11). B, a Coomassie Blue-stained gel showing the purified His-CK2 α' and β subunits. The asterisks indicate the recombinant CK2 subunits. C, mapping SSRP1 protein-protein interactions with hSPT16 and CK2 in cells. RKO human colorectal carcinoma cells were stably transfected with FLAG-N-SSRP1, FLAG-C-SSRP1, or vector control. Whole cell lysates were used in IPs using the anti-FLAG antibody. FLAG-N-SSRP1 interacts with endogenous hSPT16 (top panel), FLAG-C-SSRP1 interacts with endogenous CK2 α' (third panel from top), and can pull down p53 Ser-392 kinase activity as seen by an *in vitro* IP kinase assay using p53 C-terminal aa 311–393 as a substrate (bottom panel). IP reactions were run on SDS-PAGE and visualized by Western blotting. The dots indicate the IgG heavy and light chains. D, model of the CK2-hSPT16-SSRP1 interaction based upon the above protein-protein interaction assays. We speculate that two FACT heterodimers are bound per CK2 heterotetramer, because the apparent molecular mass of the recombinant complex *in vitro* is near 700 kDa (Fig. 1).

because their levels were equal in the nuclear extract (lanes 1 and 2). Next, p53 Ser-392 kinase activity was measured using a WB kinase assay with an α Ser-392 antibody (Fig. 6B, bottom panel). First, the p53 Ser-392 kinase activity is increased in the nuclear extract after UV irradiation ~3-fold as measured by densitometry. Second, the activity in the 0.5 M KCl fraction is also stimulated, as would be expected due to the increase in CK2 in this fraction after UV irradiation. A co-immunoprecipitation using an α SSRP1 antibody shows that SSRP1, hSPT16, and CK2 α' , and p53 Ser-392 kinase activity are associated in the 0.5 M KCl fraction after UV treatment (Fig. 6C). Because

the P11 0.5 M fraction is the only fraction in which hSPT16, SSRP1, and CK2 co-exist (data not shown), this suggests that these proteins form a complex in response to UV irradiation.

DISCUSSION

We previously identified a UV-responsive p53 Ser-392 kinase activity from F9 and HeLa cells and found that it contained protein kinase CK2 in complex with the chromatin-associated factors, hSPT16 and SSRP1 (26). There is little known concerning the biochemical properties of the hSPT16-SSRP1 heterodimer, and few reports have identified other interacting

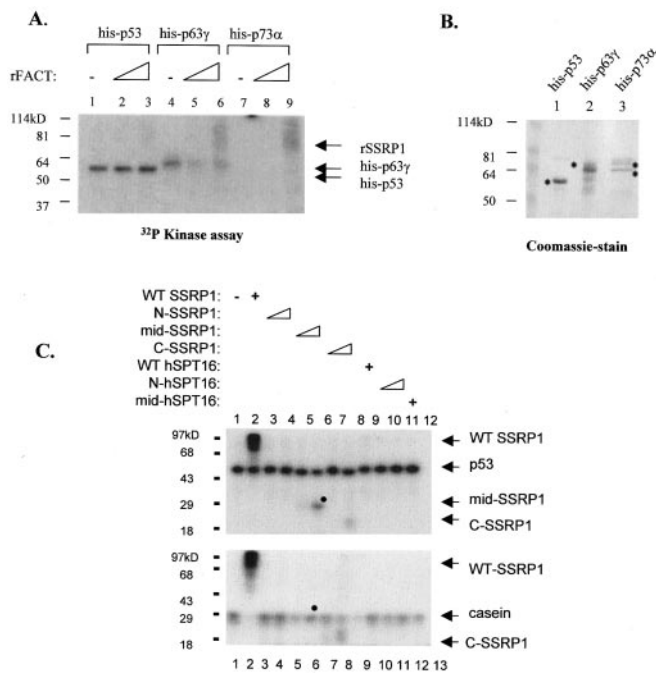


FIG. 4. SSRP1 and hSPT16 influence the substrate specificity of CK2. A, the p53 family member p63 γ , but not p73 α , is phosphorylated by CK2, although its phosphorylation is inhibited by SSRP1 and hSPT16. *In vitro* kinase reactions were done for 30 min using [γ - ^{32} P]ATP and using either 50 ng of His-p53, 150 ng of His-p63 γ , or 500 ng of His-p73 α as substrates. rFACT indicates recombinant SSRP1 and hSPT16 incubated together, titrated at 15 ng, and 30 ng of total protein. B, Coomassie-stained SDS-PAGE of the substrates used in panel A (~1 μ g of each protein was loaded). The asterisks indicate the proteins, and His-p73 α exists as two polypeptides. C, a radioactive kinase assay was done as above with either His-p53 (100 ng, top panel) or casein (1 μ g, bottom panel). CK2 was incubated along with the various SSRP1 and hSPT16 proteins as described under "Experimental Procedures." The dots indicate that casein and the mid-SSRP1 construct are both phosphorylated by CK2 and have equal migration on SDS-PAGE. Thus, although casein phosphorylation in lane 6 is inhibited, the signal is actually due to mid-SSRP1 (see lane 6, compare the top and bottom panels).

partner proteins (38, 40–42). In this study, we describe an initial biochemical analysis of hSPT16-SSRP1 and its association with CK2. We report that these proteins interact with each other via non-overlapping domains *in vitro* and in cells and that upon binding to CK2, hSPT16 and SSRP1 may induce a conformational change in the kinase such that it preferentially recognizes p53 as a substrate. We also present evidence that the association of the CK2-hSPT16-SSRP1 complex is increased following UV irradiation.

Biochemical Mechanism of the hSPT16-SSRP1-CK2 Kinase Complex—hSPT16 and SSRP1 form a stable heterodimer in cells that is conserved from yeast to mammalian systems (31, 37, 43) and is important for transcription and replication through chromatin. The biochemical role for this heterodimer (called FACT in mammalian cells) is not clear, although it may involve binding to histones H2A and H2B thereby disrupting the nucleosome octamer (31) and/or physically manipulating DNA by inducing negative supercoils (37). However, basic questions remain, such as the nature of their mutual protein-protein interaction domains. Here we have used truncation mutants of hSPT16 and SSRP1 to map their respective binding domains both *in vitro* and in cells (Figs. 2 and 3C). Using GST fusion protein binding assays we find that hSPT16 binds to the N terminus of SSRP1 and that SSRP1 binds to the central region of hSPT16 (Fig. 2, B and D). In cells, we have confirmed the N-SSRP1 and hSPT16 interaction by co-IP (Fig. 3C, top

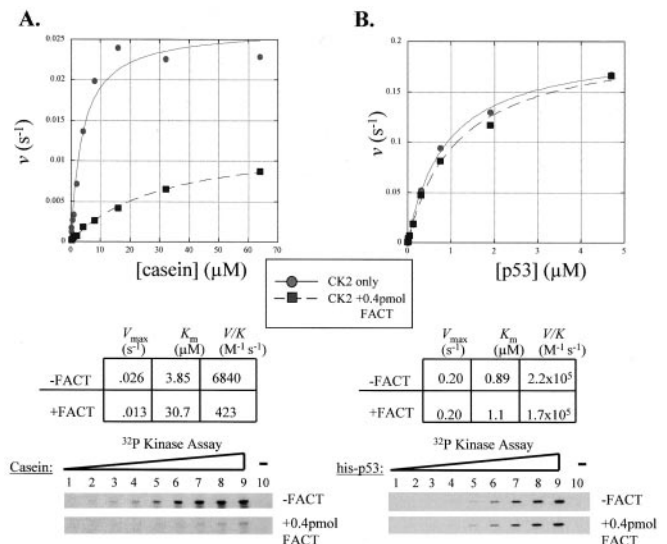


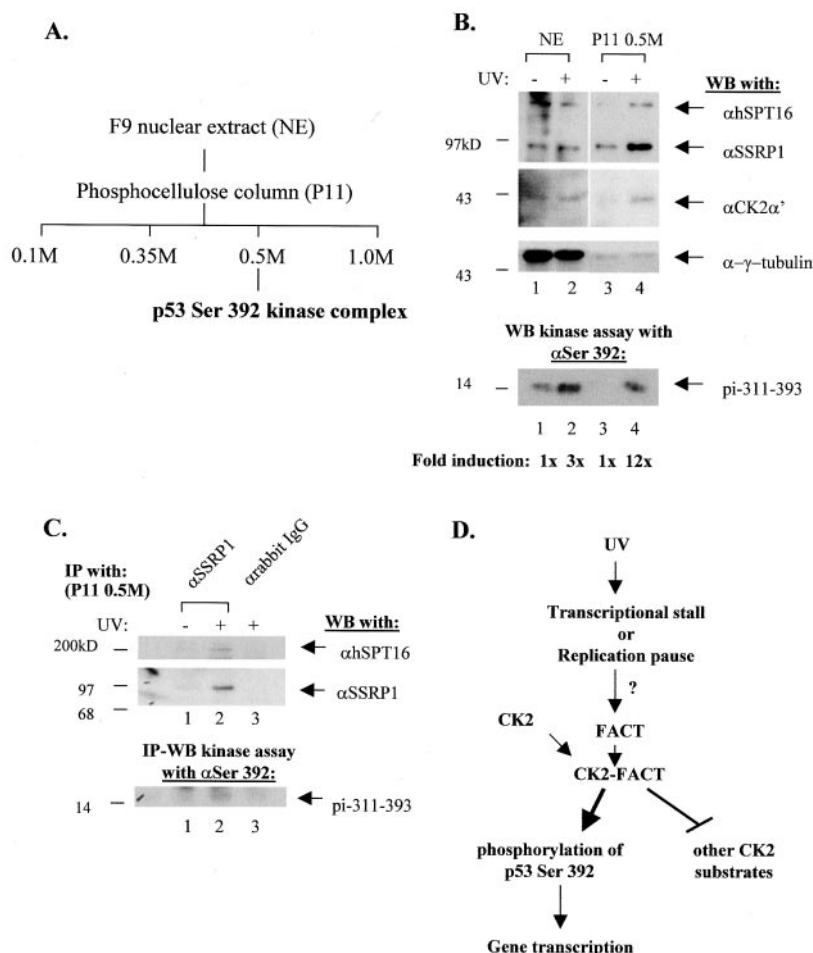
FIG. 5. Kinetic analysis of the substrate specificity of CK2 in the presence and absence of FACT. A, FACT inhibits the phosphorylation of casein by CK2. 0.5-h radioactive *in vitro* kinase reactions were carried out using CK2 (0.5 unit) with or without 4 pmol of FACT (2 pmol of FLAG-hSPT16 and 2 pmol of His-SSRP1). The kinase reactions were run by SDS-PAGE (bottom panel), the phosphorylated casein bands were cut from the gel, and the incorporation of [γ - ^{32} P]ATP was quantified by scintillation counting. The reaction velocity was then plotted versus substrate concentration (top panel), and the data points were fit to the Michaelis-Menten equation to obtain V_{max} , K_m , and V/K values (middle panel). The data is an average of two experiments. B, phosphorylation of p53 by CK2 is not affected by FACT. Kinetic analysis was performed as above except that His-p53 was titrated as indicated.

panel). Interestingly, Brewster *et al.* (43) demonstrated in *Saccharomyces cerevisiae* that POB3 (human SSRP1) failed to interact with the N terminus of SPT16, consistent with our finding that it interacts with the central domain of hSPT16 and suggesting that the protein-protein interaction domains are conserved between yeast and mammals.

Although hSPT16 and SSRP1 are a conserved heterodimer, they have also been shown to interact with other proteins, thereby providing them with additional functions. For example, in *S. cerevisiae* they bind to the catalytic subunit of DNA polymerase α (38), the histone acetyltransferase complex NuA3 (41), and have independently been found to elute from a size exclusion column at ~400 kDa (43), larger than the predicted size of 180 kDa for the yeast proteins. In mammals, an interaction has been described between these proteins and the transcription initiation factor TFIIE (42). Here we show that the molecular mass of the recombinant CK2-FACT complex is ~700 kDa, the same size as the purified native complex (Fig. 1) (26). This suggests that there are multiple copies of some or all of the proteins in the complex. For example, in Fig. 3D we present a model in which there are two FACT heterodimers for every CK2 heterotetramer, which would make the predicted size of the complex equal to 580 kDa and similar to the observed molecular mass as judged by gel filtration analysis. However, we cannot rule out that other proteins may be part of the native complex in cells. In fact, this appears to be the case in *S. cerevisiae*, demonstrated by a recent study that identified several transcription elongation complexes, including one containing CK2, SPT16, POB3 (yeast SSRP1), and CHD1, a chromodomain-helicase-DNA binding family member (44).

In this report we have performed preliminary mapping of the CK2-hSPT16-SSRP1 interactions again using GST fusion protein association and co-IP assays. We find that CK2 binds directly to the central region and C terminus of SSRP1 and to the N terminus of hSPT16 (Fig. 2, C and D). In a similar

FIG. 6. **p53 Ser-392 kinase is activated after UV treatment.** A, outline of F9 nuclear extract fractionation by phosphocellulose (P11) column chromatography that is used to purify the p53 Ser-392 kinase complex from the 0.5 M KCl fraction. B, the p53 Ser-392 kinase complex components are enriched in the P11 0.5 M KCl fraction after UV. WB analysis of nuclear extract and P11 0.5 M KCl fraction from F9 cells treated with or without UV irradiation (20 J/m²), showing that hSPT16, SSRP1, CK2 α' , but not γ -tubulin are increased in the 0.5 M fraction, although their protein levels are equal in $-/+$ UV-treated nuclear extracts. Bottom panel: the p53 Ser-392 kinase increases in specific activity after UV, and this UV-responsive activity is found in the P11 0.5 M fraction. WB kinase assay using the α Ser-392 antibody showing that the Ser-392 kinase increases specific activity in nuclear extracts $\sim$ 3-fold after UV as measured by densitometry (see "Experimental Procedures" for details). C, hSPT16, SSRP1, and CK2 associate in the UV-treated P11 0.5 M fraction. Anti-SSRP1 was used for co-IP of the p53 Ser-392 kinase complex components from the $-/+$ UV-treated P11 0.5 M fraction (30 μ g of total protein). Bottom panel: anti-SSRP1 was also used for a IP-kinase assay using the p53 C-terminal 311–393 peptide (100 ng) as a substrate, and phosphorylated product was detected by WB with the α Ser-392 antibody. D, model for the UV activation of the CK2-hSPT16-SSRP1 complex and its subsequent phosphorylation of p53 Ser-392 at the expense of other cellular CK2 substrates.



experiment, the CK2 α' subunit binds to both hSPT16 and SSRP1 and the CK2 β subunit binds strongly only to SSRP1 (Fig. 3A). Likewise, in cells, CK2 binds to the C terminus of SSRP1 (Fig. 3C). Therefore, the proteins interact via non-overlapping regions, consistent with the hypothesis that they form a complex.

Using steady-state kinetic analysis of the CK2 kinase reaction, we show that hSPT16 and SSRP1 have different effects on CK2 depending upon the substrate being phosphorylated. For example, casein phosphorylation by CK2 is severely inhibited by inclusion of hSPT16 and SSRP1 into the reaction, exhibiting a decrease in the maximal reaction rate ($V_{\max}$) and increase in K_m , with a corresponding decrease in enzyme efficiency (V/K) (Fig. 5A). In contrast, p53 phosphorylation at Ser-392 is not affected (Fig. 5B). The drop in $V_{\max}$ indicates that hSPT16 and SSRP1 bind to a site on CK2 that does not overlap the substrate binding pocket, and the increase in K_m indicates that casein no longer binds to the substrate binding pocket efficiently, perhaps reflecting a change in the conformation of the kinase. Furthermore, the sharp decrease in enzyme efficiency seen when CK2 phosphorylates casein while complexed with hSPT16 and SSRP1 suggests that in cells p53 is preferentially being phosphorylated at the expense of other CK2 substrates. This substrate specificity even extends to the p53 family member p63 γ , whose phosphorylation by CK2 is inhibited by SSRP1 and hSPT16 (Fig. 4A). Also, based on our mutational analysis, the C-terminal two-thirds of SSRP1 are necessary for influencing the substrate specificity of CK2 (Fig. 4C, bottom panel, lanes 5–8), and although full-length hSPT16 can also affect the substrate specificity (Fig. 4C, bottom panel, lane 9), the truncations do not have this ability alone (lanes 10–13).

DNA Damage-induced Activation of the CK2-hSPT16-SSRP1 Complex—Although Ser-392 was one of the first identified phosphorylation sites on p53 in cells (45), its role in p53 biology is still uncertain. It has been proposed to enhance the transcription potential of p53 based on *in vitro* results in which p53 phosphorylated at this site leads to increased sequence-specific DNA binding (25). Indeed, several experimental approaches in cells and in mice have supported this notion (26, 28, 29, 46), contributing to the hypothesis that phosphorylation of p53 fine-tunes the protein to respond to specific stresses. Ser-392 of p53 fits this model as well, because it is phosphorylated specifically after UV but not γ irradiation in multiple cell types (3, 4). Thus it is of particular interest in the identification of the kinase that targets this site *in vivo* after UV irradiation.

Here we report that phosphorylation of p53 Ser-392 increases $\sim$ 3-fold in F9 cells following UV irradiation as measured by an *in vitro* WB-kinase assay (Fig. 6B, bottom panel). This UV-responsive kinase activity fractionated from the phosphocellulose column at 0.5 M KCl (Fig. 6A and bottom panel of 6B), and by WB analysis we detected dramatically increased protein levels of CK2, hSPT16, and SSRP1 (Fig. 6, B and C). This increase was not due to misloaded samples, because a WB for γ -tubulin shows that the total protein levels are equal in this fraction (Fig. 6B) and protein measurement by Bradford assay detected equal levels of protein (data not shown). Thus the kinase complex components change chromatographic properties identically, perhaps reflecting an increase in the CK2-hSPT16-SSRP1 complex following DNA damage. The stoichiometry of these components appears to be critical, because

elevating the SSRP1 level alone did not affect p53 Ser-392 phosphorylation³ nor did it affect p53 activity (47).

With the evidence in our studies, we can begin to build a model for how the CK2-hSPT16-SSRP1 complex regulates phosphorylation of p53 Ser-392 following DNA damage (Fig. 6D). First, binding of FACT to CK2 *in vitro* is not sufficient to increase the specific activity of CK2 toward p53 (Fig. 5B), so then why is this association needed for p53 Ser-392 phosphorylation? The reason is that the association modulates CK2 probably through a conformational change (Fig. 5A) such that it preferentially targets p53 Ser-392 at the expense of the many other CK2 substrates in cells, thus giving specificity to a kinase that is normally one of the most unspecific protein kinases known (35). In this regard, an increase in the CK2-hSPT16-SSRP1 complex following DNA damage signals would lead to a corresponding decrease in free CK2 pools in the cell. This would then result in a decrease in non-p53 cellular substrates for CK2, such that the CK2-hSPT16-SSRP1 complex now phosphorylates more p53 molecules per cell, thereby leading to the increase in p53 Ser-392 phosphorylation that we see following UV irradiation (Fig. 6B).

The mechanism for how the CK2-FACT complex assembles after DNA damage is not yet clear. We speculate that sites of DNA damage may be a trigger for bringing these proteins together. This hypothesis is based on the fact that SSRP1 is a high mobility group-box-containing protein that preferentially recognizes cisplatin-modified DNA (39). Furthermore, Yarnell *et al.* (48) used *in vitro* gel-mobility shift assays to show that the ability of SSRP1 to bind damaged DNA was increased by the addition of hSPT16. Therefore it is possible that, in addition to their role in transcription and replication, hSPT16 and SSRP1 may play a role in the cellular DNA damage response and may lead to preferential binding to CK2. This will be an area of future study.

Finally, although this study demonstrates that the CK2-hSPT16-SSRP1 complex preferentially targets p53, it is likely that the complex targets other unknown but important proteins as well. In accordance with this notion is that the CK2-CHD1-hSPT16-SSRP1 interaction has recently been found in *S. cerevisiae*, using a low stringency affinity purification to identify regulators of transcription elongation (44). This finding implies that this complex may regulate non-p53 substrates, because there is no yeast *p53* gene, and that hSPT16 and SSRP1 may themselves be regulated by CK2.

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³ S. Zeng, D. Keller, and H. Lu, unpublished observations.