IDENTIFICATION AND CHARACTERIZATION OF A UV-RESPONSIVE P53 SERINE 392 KINASE COMPLEX

by

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ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia related
C-	carboxy-terminus
CDC 68	cell division-cycle mutant #68 (same as yeast SPT16 gene)
CDK	cyclin-dependent kinase
CK2	casein kinase 2
CP	CDC68/POB3 (yeast heterodimer of SPT16 and SSRP1)
DUF1	DNA unwinding factor 1 (*Xenopus* heterodimer of SPT16 and SSRP1)
FACT	facilitates chromatin transcription (human heterodimer of hSPT16 and SSRP1)
FT	flow-through
GST	glutathione-S-transferase
H1	histone H1
HIPK2	homeodomain-interacting protein kinase 2
His	6x histidine tag
HMG	high mobility group
hSPT16	human homolog of yeast suppressor of Ty insertion mutations
IgG	immunogloblin type G
IP	immunoprecipitation
IR	ionizing radiation
K_m	Michaelis-Menten constant
MDM2	gene amplified on the mouse double minute chromosome
N-	amino terminus
NE	nuclear extract
SSRP1	structure specific recognition protein 1
P11	phosphocellulose column
p14ARF	p14 alternate reading frame
p38MAPK	p38 mitogen-activated protein kinase
p300/CBP	two highly homologous proteins encoded by separate genes: p300 and creb-binding protein
PCAF	p300/CBP associated factor
pi-p53	phosphorylated p53
POB3	polymerase-one binding protein (yeast SSRP1 homolog)
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
UV	ultraviolet
V_{max}	maximal reaction velocity
WB	Western blot
WT	wildtype

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ABSTRACT

The p53 tumor suppressor protein is modified in cells by phosphorylation on most of its serine and threonine residues often in a cell stress-dependent manner. These modifications are generally believed to enhance p53's ability to bind sequence-specific DNA and to increase its protein half-life. Serine 392 (out of 393 amino acids on the human p53 protein) is phosphorylated in cells following UV but not γ irradiation, and phosphorylation at this site *in vitro* enhances p53's sequence-specific DNA binding and has been shown in several *in vivo* models to increase p53's transactivation function.

To determine how UV leads to increased p53 phosphorylation at Ser 392 and in order to characterize a potentially novel UV-p53 pathway, this thesis dissertation seeks to identify the kinase responsible for this phosphorylation and to understand how it is regulated by DNA damage using biochemical, cellular, and molecular biological approaches.

Here a purification from cells is described of the UV-responsive p53 Ser 392 kinase using mouse F9 or human HeLa cells. The kinase elutes from gel filtration chromatography at approximately 700kD, and proteins co-eluting with this activity were sequenced by mass spectrometry and identified as protein kinase CK2 and the chromatin-associated factors, hSPT16 and SSRP1. That these proteins form a high molecular weight complex is confirmed by co-immunoprecipitation, co-elution profiles from column chromatography, and by direct binding *in vitro*. Interestingly, though the purified p53 Ser 392 kinase contains protein kinase CK2, it does not efficiently phosphorylate typical CK2 substrates other than p53. Furthermore it is shown *in vitro* that hSPT16 or SSRP1 alone inhibit CK2's ability to phosphorylate the substrate casein, while not affecting p53 Ser 392 phosphorylation.

The protein-protein interaction domains between CK2, hSPT16, and SSRP1 are studied *in vitro* and in cells using truncation mutations and it is shown that they bind to each other via non-overlapping regions, supporting the evidence that they bind as a protein complex. Steady state kinetic analysis of CK2's kinase activity in the presence and absence of hSPT16 and SSRP1 reveals that these proteins influence the substrate specificity of the kinase possibly by changing its conformation such that it preferentially recognizes p53. Also, CK2, hSPT16, and SSRP1 are affected by UV irradiation such that they found together more abundantly by column chromatography, indicating perhaps that they assemble after DNA damage. The specific activity of the p53 Ser 392 kinase increases as well after UV, due in part perhaps to p38 mitogen-activated protein kinase (p38MAPK), which has been shown to activate CK2. A specific inhibitor of this kinase reduces UV-induced p53 Ser 392 phosphorylation in cells, and immunoprecipitates of p38MAPK from cellular extracts contain p53 Ser 392 kinase activity.

Thus our data supports a role for direct activation of the CK2-hSPT16-SSRP1 complex by UV light and thus p53 Ser 392 phosphorylation, and also supports a role for p38MAPK, possibly by increasing the specific activity of this complex.

CHAPTER ONE

INTRODUCTION

A. The p53 tumor suppressor.

The tumor suppressor protein p53 is a critical mediator between a wide variety of cell stress signaling pathways and gene expression. Cell stressors which activate p53 include DNA damaging agents such as irradiation and chemical mutagens, oncogenes, hypoxia, and reactive oxygen species (reviewed in 46, 129). Once these stresses impinge upon the cell, p53 is stabilized at the protein level and its transcriptional activity increases. The activated p53 protein induces transcription of target genes by binding to a specific DNA sequence found in the promoters of genes involved in cell growth arrest and programmed cell death, or apoptosis (46). Thus p53 provides a crucial mechanism for preventing the cell from dividing or surviving if its genome has sustained DNA damage that could potentially lead to aberrant cell growth and tumorigenesis.

It is necessary for a cell to escape from normal growth controls in order to develop into a tumor. Thus, if the p53 gene itself is mutated, then the cell has a distinct growth advantage. In fact, on average over half of all human tumors have a mutation in the p53 gene (60). Of these mutations, the majority are inactivating missense mutations located in the sequence-specific DNA binding domain, highlighting the importance of this region for p53 function (Figure 1.1A) (reviewed in ref. 87). Other critical regions of the p53 molecule include the amino-(N)-terminal acidic transactivation domain that makes contacts with basal transcription factors and co-activators (36, 137), the carboxy-(C)-terminal tetramerization domain (163), and the extreme C-terminal regulatory region that is thought to regulate the sequence-specific binding activity of the core

domain and can independently bind non-specifically to DNA (Figure 1.1B) (69, 71, 75, 95).

There are over a hundred described genes activated by p53, many of which lead to cell cycle arrest, thus giving the cell a chance to repair DNA damage, or lead to apoptosis if the damage is too severe (reviewed in ref. 160). Many other targets of p53 have been identified as well, including genes involved in DNA repair, maintenance of genomic stability, and prevention of angiogenesis (160). The best characterized example of how p53 induces cell cycle arrest is in the case of $p21^{WAF1/CIP1}$ (30, 35, 59). p21 is a cyclin-dependent kinase (CDK) inhibitor that binds to and inactivates several cyclin/CDK complexes at both the G1 to S phase transition and the G2 to M phase transition (15, 169). When active, the cyclin/CDK complexes phosphorylate the retinoblastoma protein (Rb) family members, resulting in release of the E2F transcription factor which in turn activates transcription of many genes involved in replication and nucleotide biosynthesis, as well as CDK2 and cyclin E, forming a positive feedback loop (Figure 1.2) (reviewed in ref. 123).

Depending upon the inducing signal and type of cell, p53 will not promote cell cycle arrest but instead will promote apoptosis (160). A large number of proapoptotic genes are activated by p53, many of which are connected with the caspase cascade. For example, Bax (116), PIG3 (133), PUMA (121), p53AIP1 (126), Killer/DR5 (150), and caspase 6 (109) are such genes. The caspases are proteases that cleave cellular substrates and thereby physically break down the cell in response to death signals (153).

With over one hundred known transcriptional targets of p53, recent work has begun to look at the specific tissues in which these genes are activated. For example, the el-Deiry lab (34) has found distinct sub-tissue localization for two p53 target genes that

apparently have identical downstream effects by activating the caspase cascade, and therefore apoptosis. After treating mice with ionizing irradiation and performing histological sections of the spleen, they found that the proapoptotic gene and p53 target gene Bid was expressed only in the "red pulp" whereas another p53 target gene, PUMA, was expressed only in the "white pulp". Moreover, the caspase cascade activator Bax was expressed in neuronal tissues after mice were irradiated, whereas Killer/DR5 was expressed exclusively in the thymus.

Because p53 is a potent inducer of cell growth arrest and apoptosis, its own expression is tightly controlled so that the cell does not stop growing or die under normal circumstances. The mechanisms by which this control occurs is through regulation of its protein stability and its translation (46). The stability of the p53 protein is controlled by the MDM2 protein (61, 90), which is an E3 ubiquitin ligase enzyme (67). Along with E1 and E2 enzymes, MDM2 catalyzes the transfer of a chain of ubiquitin proteins onto C-terminal lysine residues of p53, targeting p53 for rapid degradation by the proteosome pathway (Figure 1.3). To ensure that p53 is kept under tight control, MDM2 itself is a target gene for p53, thus forming a built-in auto-inhibitory feedback loop (6, 117, 167). The functional link between these proteins is clearly shown by knockout mouse models in which MDM2 homozygous null mice are embryonic lethal, though double knockout MDM2-p53 mice survive (78, 118). This striking finding indicates that without MDM2 to keep the p53 protein in check, the organism cannot survive p53's growth suppressive effects. In addition to its ubiquitin ligase activity, MDM2 can physically block p53's ability to transactivate genes through binding to and masking of its N-terminal transactivation domain (20, 128). Thus there are at least two ways that MDM2 can inhibit p53 function: control of protein stability

and activity.

MDM2 itself is controlled by multiple mechanisms so that p53 can transactivate its target genes at the appropriate time after cellular insults. One mechanism which is activated by oncogenic stimuli or aberrant growth factor signaling is via the tumor suppressor protein, p14ARF, which binds to and sequesters MDM2 away from p53 in the nucleolus (Figure 1.2) (134, 152, 179). Another is through phosphorylation of p53 and MDM2, which serves to block binding of MDM2 to p53, thus leading to an increase in p53 protein levels (see **section B**) (17, 19, 145, 157). The stabilized and activated p53 can now bind to the p53 response element (p53RE) in promoters of target genes via its DNA binding domain located in the core of the protein (160).

B. p53 posttranslational modifications.

The p53 protein is highly modified in cells by phosphorylation, acetylation, ubiquitination, sumoylation, ribosylation, and others (reviewed in ref. 2). These modifications occur outside the core DNA binding domain on both the N- and C-termini (2), suggesting that these domains and their reversible modifications play a role in regulating p53 transactivation potential and DNA-binding activity. In fact, p53 is regulated in this manner. For example, phosphorylation on the N-terminal MDM2 binding domain results in the inability of MDM2 to bind and degrade p53, thus leading to increased p53 half life (17, 19, 145, 157). On the regulatory C-terminus, both phosphorylation and acetylation combine to give more efficient sequence-specific DNA binding (51, 71, 99, 140).

Moreover, many of the posttranslational modifications are stress-activated thereby providing a mechanism to turn a latent, unmodified p53 into a functioning

transcription factor (2). The development of phospho- and acetyl-specific antibodies against many of these sites has allowed the study of p53 modifications to expand rapidly in recent years. The growing consensus is that cellular stresses activate specific patterns of phosphorylation and acetylation in a time-dependent and stress-dependent fashion. For example, the N-terminal residues Ser 6, 9, and 15 undergo phosphorylation in response to ionizing irradiation (IR) within 30 min after treatment (63, 148), whereas the same sites are phosphorylated less rapidly but remain phosphorylated for a longer duration in response to ultra-violet irradiation (UV) (2). When Ser 20 was examined after nitric oxide (NO) and adriamycin treatments, phosphorylation was detected after a half hour with NO compared with 3 hours for adriamycin (122). At the C-terminal, Ser 392 phosphorylation occurs after UV or cisplatin treatment, but not after IR or etoposide (80, 105). Also at the C-terminal, there are five lysine residues which are acetylated in cells in a similar fashion after various forms of stress (74), perhaps reflecting the fewer number of acetyltransferases that target p53.

All told, there are fourteen reported phosphorylation sites and five reported acetylation sites on p53, but slightly fewer than that number of signaling pathways that target p53 due to the fact that some pathways target multiple sites (Figure 1.4) (2). One of these pathways is that of the ATM kinase (for ataxia telangiectasia mutated) which is rapidly activated by IR and signals to p53 through two N-terminal serine residues (5, 17). Ser 15 is a direct substrate (145), while Ser 20 is possibly phosphorylated by an ATM-Chk2 kinase cascade (8, 64). Both appear to play a role in stabilization of p53 by blocking its interaction with MDM2. Ser 15 is also targeted after UV by the ATR kinase (for ataxia telangiectasia related) that also targets Ser 37, both of which contribute to p53 stability (154). A recently identified phosphorylation site, Ser 46, provides a

glimpse of how refined the connection might be between phosphorylation and p53 function. Strikingly, Ser 46 in human cells was required for induction of p53-dependent apoptosis but not for cell growth arrest (126), and was required for the specific induction of the pro-apoptotic gene p53AIP1 but not other apoptotic genes such as PIG3. Independently, a Ser 46 kinase was identified as the homeodomain-interacting protein kinase 2 (HIPK2) (26, 65), though one of these studies showed that p53 phosphorylated by HIPK2 could induce cell growth arrest. Nevertheless, these studies raise the possibility that p53 may activate certain target genes in phosphorylation-specific ways.

Acetylation of p53 occurs exclusively on the C-terminus: the p300/CBP acetyltransferase predominantly targets Lys 373 and 380, while PCAF (p300/CBP associated factor) targets Lys 320 (2). These enzymes apparently serve multiple functions for p53. Acetylation of lysine residues may enhance p53's DNA binding activity (51) and additionally binding of acetyltransferases to p53 recruits them to promoters where they contribute to an open-promoter complex by acetylating histones and recruiting other co-activators (146). Interestingly, the p53 C-terminal lysines are also targets of ubiquitination mediated by MDM2 (61, 67, 90), thus potentially setting up a direct competition between stabilizing p53 via acetylation and degrading it via ubiquitination (Figure 1.4). This is supported by the fact that MDM2 can directly inhibit acetylation by p300/CBP and PCAF (Figure 1.4) (74, 76, 88).

C. Serine 392 phosphorylation.

Ser 392 along with Ser 315 were the first two phosphorylated sites identified on p53 (141). In that study, murine Ser 389 (equivalent to human Ser 392) phosphorylation

increased in NIH 3T3 cells following transformation with a temperature-sensitive mutant of the Simian virus 40 (SV40) T antigen and grown at the permissive temperature, but not when cells were grown at the nonpermissive temperature. However, this increase in phosphorylation was not repeated by Meek *et al.* (113) using NIH 3T3 cells either not transformed or transformed with wildtype SV40 T antigen.

Ser 392 was also the first site for which a p53 kinase was found, and was identified as casein kinase 2 (CK2) (114). In their study, Meek *et al.* (114) hypothesized that CK2 was a p53 kinase because both p53 and CK2 were involved in cell growth control and also because CK2 had a broad substrate specificity that included nuclear oncoproteins functionally associated with p53. They used recombinant mouse p53 and a highly purified CK2 preparation from rat testes and determined that phosphorylation was occurring on Ser 389 (human Ser 392). Additionally they showed using phosphocellulose chromatography that the purified CK2 and the endogenous p53 Ser 389 kinase activity from NIH 3T3 cells co-eluted. The consensus target sequence for CK2 is serine or threonine residues that are positioned within or on the N-terminal side of clusters of highly acidic amino acids (1). p53 has a sequence containing a serine residue flanked by aspartic acids, KVGPDSD (amino acids 387 to 393 of the human sequence, mouse and human sequences are identical), consistent with the idea that CK2 targets this site.

A clue to the functional role of Ser 392 phosphorylation by CK2 came with the discovery that the p53 C-terminal domain negatively regulates sequence-specific DNA binding (71). In this study Hupp *et al.* (71) found that recombinantly expressed p53 binds weakly *in vitro* to DNA oligomers containing the p53-responsive element (p53RE). Using gel mobility shift assays, they discovered that addition of the PAb421

monoclonal antibody directed against a C-terminal epitope or deletion of the last 30 amino acids of the protein dramatically enhanced DNA sequence-specific binding by recombinant p53. This result was confirmed in cells by microinjection of the PAb421 antibody into murine fibroblasts containing wildtype p53 and showing that it could activate a latent pool of p53 in the cells in the absence of DNA damage (70). This result suggested that the normal role of the C-terminal domain is to keep p53 in a latent state by inhibiting the central DNA binding domain. Furthermore, Hupp *et al.* (71) discovered that phosphorylation of p53 by CK2 at Ser 392 recapitulated the relief of inhibition by the C-terminal antibody or the C-terminal deletion *in vitro*, suggesting that this auto-inhibition may be a mechanism in the cell to regulate the DNA binding activity of p53. A biophysical study by Sakaguchi *et al.* (140) suggested that increased sequence-specific DNA binding by Ser 392 phosphorylated p53 may be due to increased stabilization of the p53 tetramer. They showed that p53 peptides phosphorylated at Ser 392 formed more thermodynamically stable tetramers than the unphosphorylated peptide, Ser 315 phosphorylated p53, or p53 acetylated at four lysine residues. Their data fit with a model in which p53 tetramer stabilization by Ser 392 phosphorylation resulted from the formation of new intra- or intermolecular polar interactions such as hydrogen bonds.

Conflicting reports have emerged as to the importance of Ser 392 phosphorylation for p53 function in cells. First, in tumors p53 has not been found with mutations at Ser 392 or nearly all other phosphorylation and acetylation sites (87), a result that has been taken as evidence by some that post-translational modifications play only a minimal role in p53 biology (3). However, an alternate explanation is that post-translational modifications have redundant roles in activating and stabilizing p53, such

that loss of one site does not critically impair p53 function because of compensation from other sites (2, 46). Several studies have attempted to investigate the importance of these phosphorylation sites including Ser 392 using transient transfection assays (3, 24, 41, 56, 112). Using this approach, Crook *et al.* (24) found that mutating Ser 392 to Ala in the human osteosarcoma SAOS2 cell line had no effect on p53-mediated growth suppression but in contrast impaired p53's ability to suppress ras-mediated transformation in rat embryonic fibroblasts. Taking a different approach Hao *et al.* (56) showed that when wildtype p53 is normally nonfunctional in G1-arrested NIH3T3 cells, only the p53 mutant with a Ser 389 > Glu substitution (equivalent to human Ser 392) retained both DNA binding and transactivation functions when compared with 5 other serine mutations. They hypothesized that the negative charge on glutamic acid could functionally mimic the phosphate moiety on Ser 389. These studies, while instructive, suffer from the inherent complications of overexpression experiments in which high levels of p53 mask the subtleties of p53 regulation by post-translational modifications *in vivo*.

A better model was recently described by the Jack laboratory (27), in which they generated Ser 389 > Ala knock-in mice. Preliminary studies with the Ser 389 > Ala homozygous knock-in mice demonstrated a severe deficiency in protection from the carcinogen amino-acetyl-fluorine (AAF) in the bladders (27). 14/15 of the homozygous knock-in mice ($p53^{S389A/S389A}$) developed bladder carcinomas while 0/15 Ser 389 > Ala heterozygous mice ($p53^{+/S389A}$) with one functional copy of p53 developed tumors and 15/15 p53-null mice developed tumors. Though these mice are still being characterized, the initial results that p53 Ser 392 phosphorylation is critical for p53 function *in vivo* seem intriguing.

Another characteristic of p53 Ser 392 phosphorylation was revealed by two studies using phospho-specific antibodies which showed increased phosphorylation in cells treated with UV irradiation but not with IR (80, 105). Lu *et al.* (105) showed that p53 protein levels and Ser 392 phosphorylation increased steadily to a maximum at 7 hours post-UV treatment, whereas IR induced a different pattern of p53 protein induction with no concomitant Ser 392 phosphorylation. Kapoor and Lozano (48) used rat embryo fibroblasts to show that p53 from UV-irradiated cells, once bound to DNA in a gel mobility shift assay, could be immunoprecipitated by their phospho-Ser 389 (human Ser 392) antibody. However, these studies raised a new question as to the kinase that targets this site in cells after UV irradiation, as protein kinase CK2 was not known to be activated after UV irradiation. This suggested two possibilities: either CK2 is not the UV-responsive kinase or CK2 is regulated in a previously unknown manner after UV treatment.

From the accumulated evidence, Ser 392 appears to be critically important in order to activate latent p53 fully after DNA damaging signals such as UV. Furthermore, it may be required for p53 transcriptional activity in particular tissues and after certain forms of cell stress, as evidenced by the newly described Ser 389 > Ala knock-in mouse model (18). Thus it is important to understand how this site is regulated after DNA damage, and the crucial first step is to identify the kinase that targets this site. There are three potential scenarios. First, the kinase may be part of an already well-established UV-responsive pathway; second, it may be a previously unidentified kinase that targets this site; and third, the kinase may be already known but regulated in a novel way after UV-induced DNA damage.

This thesis research project addresses the question of what is the p53 Ser 392

kinase, and its regulation in response to UV radiation. The approach we have taken to is identify the kinase is by means of a chromatographic separation of nuclear extracts from murine testicular carcinoma F9 cells and to trace the UV-responsive p53 Ser 392 kinase activity throughout the fractionation with an *in vitro* kinase assay using a phospho-Ser 392 antibody to detect the phosphorylated p53 substrate. Our results fit with the third scenario mentioned above, that is, we identified the p53 Ser 392 kinase as protein kinase CK2. Interestingly, it is regulated by novel protein-protein interactions with the transcription elongation factors hSPT16 and SSRP1. These two proteins alter CK2's substrate specificity in the complex to preferentially phosphorylate p53 over all other tested substrates. The hSPT16-SSRP1-CK2 kinase complex appears to be constitutively associated even in the absence of DNA damage, though after UV irradiation the complex formation increases, thereby providing a potential mechanism for UV-induced p53 Ser 392 phosphorylation. We and others (68, 83) have also presented evidence that the specific activity of the Ser 392 kinase increases after UV irradiation and that a potential mechanism for this increase in activity is through the UV-activated p38 mitogen-activated protein kinase (p38MAPK), that may participate in a p38MAPK-CK2 activation cascade (see **Chapter Four**).

D. CK2, hSPT16, and SSRP1.

Casein kinase 2 (CK2) is a ubiquitous serine/threonine protein kinase that generally functions as a ~130kD heterotetramer consisting of catalytic α or α' subunits and regulatory β subunits, such that the stoichiometry is either $\alpha_2\beta_2$, $\alpha'_2\beta_2$, or $\alpha\alpha'\beta_2$ for the holoenzyme (reviewed in ref. 1). It has been reported to phosphorylate over 160 substrates, many of which are involved in cell cycle-related signaling processes such as

proliferation and growth control (reviewed in ref. 52). In fact, studies from yeast and mammals show that CK2 is required for cell cycle progression (55, 132, 156) and that some human leukemias (39) and solid tumors (120) have increased levels of CK2, suggesting a role in cell cycle regulation. In contrast, other studies have shown that overexpression of CK2 inhibited the ability of Ras to transform mouse NIH 3T3 cells (62), and inducible expression of CK2 at physiological levels led to diminished cellular proliferation of human osteosarcoma U2-OS cells (159). Thus, the discrepancies in the literature suggest alternate roles for CK2 depending upon the cellular context.

CK2 has been described as "a protein kinase in need of control" (52) because it is one of the most unspecific of eukaryotic kinases, phosphorylating greater than 160 substrates *in vitro*, having the rare ability to use either ATP or GTP as phosphoryl donors, phosphorylating serine, threonine, and tyrosine residues, and using not only Mg^{2+}, but also having the ability to use Mn^{2+} or Co^{2+} as cofactors. Additionally, CK2 is not regulated by known second messengers or by phosphorylation and has constitutive activity toward most substrates *in vitro* (52). Instead, the activity of the catalytic subunits are regulated by association with the regulatory subunits which increase the kinase activity toward most substrates, though in some cases the opposite occurs (1). Also, CK2 is regulated by polybasic and polyacidic molecules such as polyamines and heparin, of which polyamines such as spermine activate CK2 in a substrate-dependent fashion, while heparin inhibits its activity (1).

Also surprisingly, CK2 is apparently activated in at least two cases by other kinases in a phosphorylation-independent manner, one of which is $p34^{cdc2}$ (11) and the other by the UV-activated p38MAPK (142). Sayed *et al.* (142) found that several cellular stresses activated CK2 and that this activation could be inhibited by pre-

treatment of cells with a specific p38MAPK inhibitor, SB203580. Additionally they showed that p38MAPK could directly bind to CK2 *in vitro* and that binding was decreased several fold by incubation with SB203580. Intriguingly, we have found evidence that the same p38MAPK inhibitor SB203580 can partially inhibit UV-induced Ser 392 phosphorylation 2-3 fold as measured by an immunoprecipitation-kinase assay, suggesting a potential p38MAPK-CK2-p53 link.

Including this report, hSPT16 and SSRP1 have been independently identified several times in different capacities, including as factors important for transcription and replication through chromatin (110, 131, 138, 165), for transcription independent of chromatin (32, 149, 161), DNA damage recognition (14, 171), and now as components of the p53 Ser 392 kinase complex that modulate the kinase activity of CK2. The two proteins are conserved in all eukaryotic species and they form a stable heterodimer in cells that is also conserved (13, 127, 131). Additionally, in *Saccharomyces cerevisiae* they are both essential for viablity (110, 165). SPT16 was initially identified in *S. cerevisiae* in a screen for suppressors of Ty insertion mutations, indicating that it was important for transcription initiation (23), and separately identified as CDC68 in a screen for cell-division cycle mutants that were required for entry into the cell cycle (135), presumably because it was required for transcription of G1 cyclins. SSRP1 was initially identified in human cells as a structure-specific recognition protein that bound to DNA modified by the anti-cancer drug cisplatin (14), and separately as T160 in mice as a protein that bound to V-(D)-J recombination signal sequences in a DNA sequence-specific manner (147).

The knowledge that SPT16 and SSRP1 functioned together as a heterodimer came later from several reports studying both yeast, *Xenopus*, and mammalian systems

(13, 127, 131). In yeast they were isolated in a screen for proteins that bound to DNA polymerase α catalytic subunit, Pol1, though at the time they were not recognized as an independently-acting heterodimer (165). This study also identified for the first time the yeast ortholog of mammalian SSRP1, POB3, for polymerase one binding protein. Thus, a link between SPT16-SSRP1 and DNA replication was established. Importantly, this study showed that SPT16 and SSRP1 bound to other proteins in the cell to carry out their functions, as this thesis project describes their interaction with CK2. SPT16 and POB3 (SSRP1) were later identified as a highly abundant, stable, nuclear complex in yeast that had the ability to associate with chromatin (13, 166). In *Xenopus*, these proteins were purified as a heterodimer that could induce negative supercoils into DNA in the presence of topoisomerase I, and was termed DUF for DNA unwinding factor (127). Immunodepletion of DUF from *Xenopus* egg extracts impaired DNA replication from exogenously added sperm nuclei or plasmid DNA. Lastly, hSPT16 and SSRP1 were purified from human cervical carcinoma HeLa cells as a heterodimer that could enhance transcription elongation by RNA polymerase II (RNAPII) through a nucleosome array *in vitro*, and were called FACT for facilitates chromatin transcription (130, 131). In this study the authors found that FACT bound to core histones H2A and H2B and that covalently crossed-linked nucleosomes disrupted FACT activity. Thus they proposed that FACT could either alter nucleosome structure or could physically remove histones H2A and H2B from the core such that RNAPII could proceed through. Genetic evidence from yeast also corroborated this association with chromatin by showing that SPT16 was important for general transcriptional activation of certain genes and could also maintain repression of other genes in a genetically similar manner to histones H2A and H2B (108, 110, 135, 138), such that deletion of the H2A, H2B, or

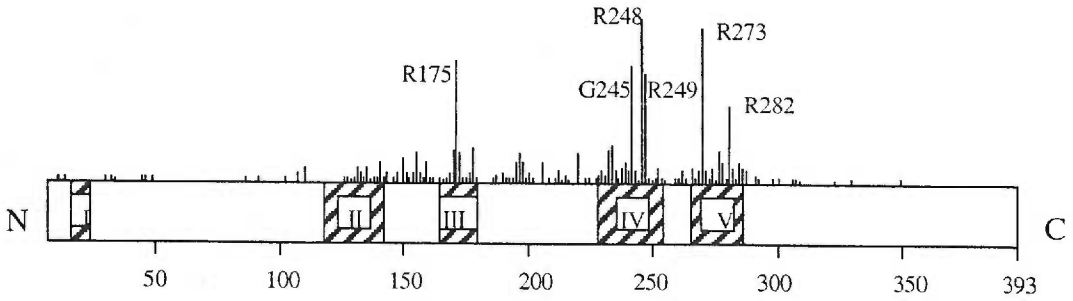
SPT16 genes resulted in enhanced expression of a similar subset of downstream genes. Furthermore, increased dosage of SPT16 and SSRP1 could rescue yeast with mutations in the chromatin remodeling factors SWI/SNF (13). This evidence establishes that SPT16 and SSRP1 play essential roles in transcription and replication by affecting nucleosome structure and potentially by physically manipulating DNA.

Though these data define a critical role for SPT16 and SSRP1, these are apparently not the only functions for these proteins. Notably, SSRP1 can bind to cisplatin-modified DNA (14), and gel-mobility shift assays show that SSRP1 can bind cisplatinated DNA with a much higher affinity in the presence of SPT16 (171). SSRP1 is a member of a large family of high mobility group proteins (HMG) which are highly abundant non-histone chromatin proteins that bind to and cause bending of DNA via their HMG box, and play architectural roles in DNA processes such as transcription, replication, and recombination by bridging together other proteins (50). Bending of DNA caused by cisplatin may then help SSRP1 bind to this particular structure via its HMG box.

Cisplatin is a potent and highly effective chemotherapeutic agent against testicular and ovarian cancers (12), and it is likely that it acts by damaging DNA thereby activating cellular repair, growth arrest, and apoptotic pathways (173). In fact, cisplatin has been shown to be an important activator of the p53 pathway (172) and can also activate phosphorylation of Ser 392 (Figure 1.5), thus providing a link between cisplatin damage, SSRP1-SPT16, and p53 activation. In this thesis, it is shown that CK2 binds to SSRP1 and SPT16 and that these proteins modulate CK2 activity such that it phosphorylates p53 Ser 392 in preference to other CK2 substrates. Based on the evidence, it is proposed that there is a link between DNA damage and p53 activation

through an SSRP1-SPT16-CK2 protein kinase complex.

Figure 1.1. *Human tumor suppressor protein p53.* **(A)** p53 mutations found in human cancer. Hatched boxes represent evolutionarily conserved regions. Vertical lines above represent the relative frequency at which mutations are found at each particular residue and are clustered in conserved regions II-IV. Several hot spots for mutations R175, G245, R248, R249, R273, and R282 are also indicated. **(B)** Structural organization of the p53 protein. Hatched boxes represent conserved regions. The p53 protein contains an N-terminal acidic transactivation domain that makes protein-protein contacts with members of the basal transcription machinery, other transcriptional co-activators such as p300/CBP, and the MDM2 negative regulator. p53 contains a core sequence-specific DNA binding domain, wherein the vast majority of p53 mutations in human tumors reside. Lastly, the C-terminus contains tetramerization and negative regulatory domains, together that form a non-specific DNA interaction domain. (Adapted from ref. 87).


R248
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350
393

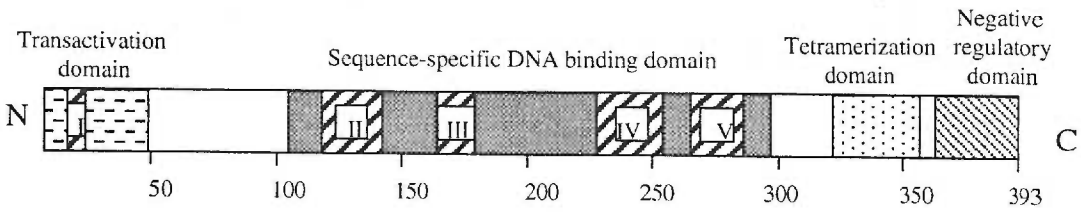

Transactivation domain
Sequence-specific DNA binding domain
Tetramerization domain
Negative regulatory domain
N
I
II
III
IV
V
C
50
100
150
200
250
300
350
393

Figure 1.2. *Schematic of several p53-induced pathways.* p53 is activated after a wide variety of intra- and extracellular stresses, and once activated induces both cell cycle arrest and apoptosis through the transcriptional activation and repression of many genes. One such gene, $p21^{WAF1/CIP1}$, is a cyclin-dependent kinase inhibitor that inhibits cyclin/CDK complexes at both G1 to S and G2/M phase transitions, that in turn normally phosphorylate the retinoblastoma (Rb) protein, thereby causing release of the cell-growth promoting E2F transcription factor. Additionally, p53 activates the expression of its negative regulator, MDM2, thus forming a negative feedback loop that serves to keep p53 in tight control. Indirectly, p53 can be activated by oncogenes and aberrant cell growth signals through E2F transcriptional activation of the p14 tumor suppressor protein, that in turn binds to and sequesters MDM2 away from p53 in the nucleolus. (Adapted from ref. 111).

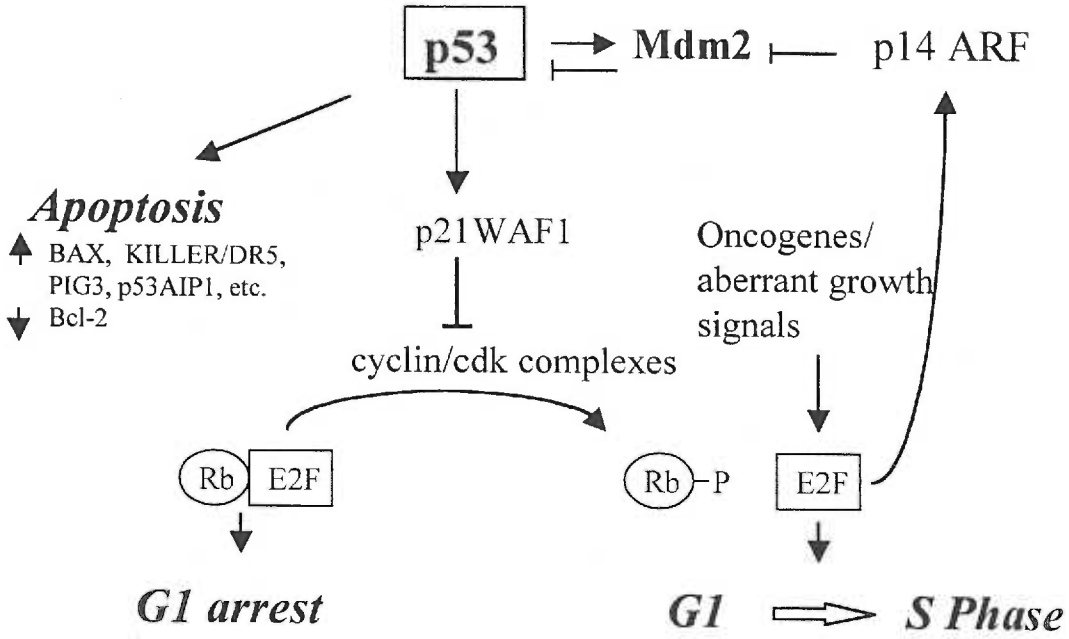

p53
Mdm2
p14 ARF
Apoptosis
BAX, KILLER/DR5,
PIG3, p53AIP1, etc.
Bcl-2
p21WAF1
cyclin/cdk complexes
Oncogenes/
aberrant growth
signals
Rb
E2F
Rb-P
E2F
G1 arrest
G1
S Phase

Figure 1.3. *Schematic of p53 control by MDM2, an E3-ubiquitin ligase enzyme.* Ubiquitination of p53 at C-terminal lysine residues by MDM2 leads to its short half-life (approx. 20 min) in normal, unstressed cells. Cellular stresses lead to phosphorylation of p53 in the MDM2-binding domain and thereby prevent association with MDM2, thus increasing p53 half-life to several hours. Recent work has shown that a de-ubiquitinase HAUSP enzyme also plays an important role in controlling the balance between stabilzation and degradation. Another critical mechanism for stabilizing p53 may be through acetylation by p300/CBP and PCAF, which target the same p53 C-terminal lysines residues that are ubiquitinated, thereby preventing ubiquitination and degradation. Interestingly, MDM2 can also inhibit this process, such that there is direct competition between the activating (p300/CBP, PCAF) and degrading (MDM2) machinery.

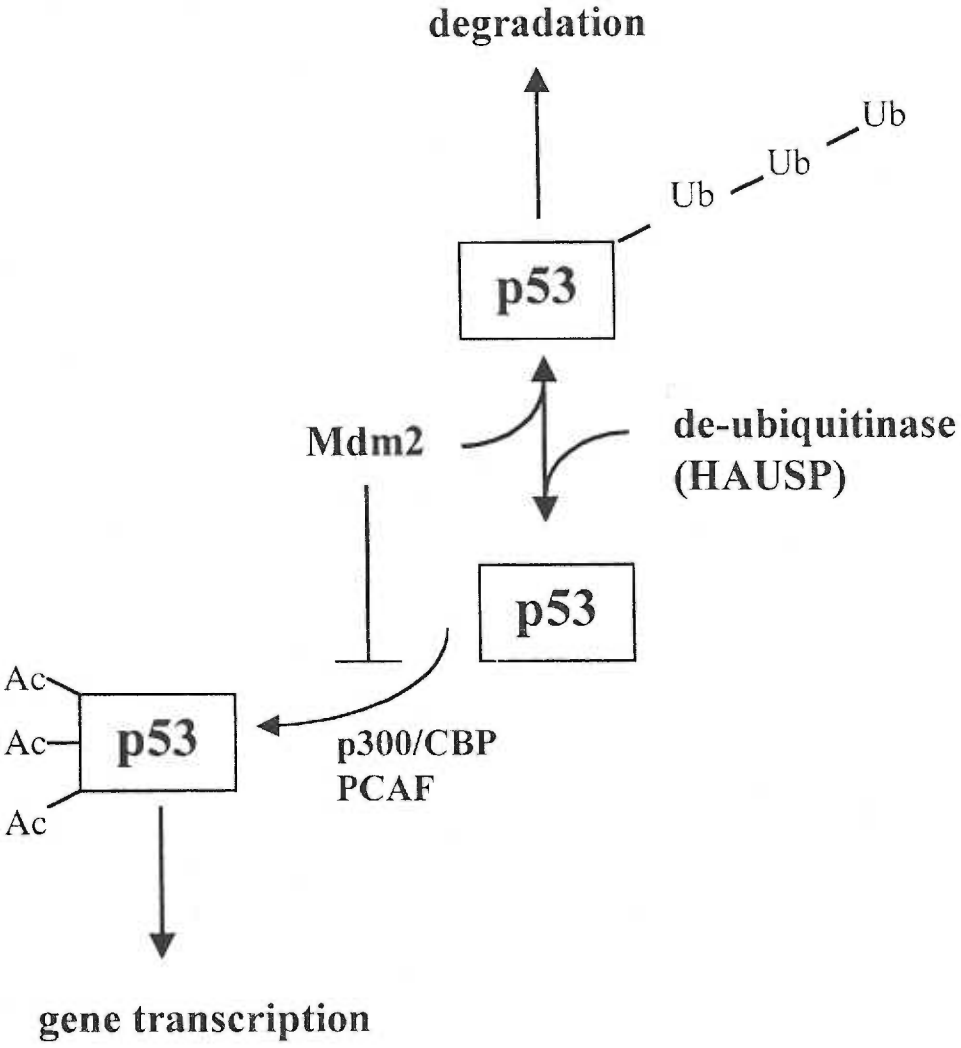

degradation
Ub
Ub
Ub
p53
Mdm2
de-ubiquitinase
(HAUSP)
p53
Ac
Ac
Ac
p53
p300/CBP
PCAF
gene transcription

Figure 1.4. *DNA damage-induced posttranslational modifications to human p53.* The bar represents the 393 amino acids of human p53 polypeptide, and the indicated regions are transactivation domains (TA), sequence-specific DNA binding (DBD), nuclear localization (NLS), tetramerization (TET), and negative regulatory domain (REG) (not to scale). The positions of known phosphorylations and acetylations are represented by ovals and squares, respectively, and listed with the appropriate amino acid identity. Documented modifying enzymes are presented. The response to ionizing radiation (IR) is believed to initiate from DNA strand breaks that are sensed directly or indirectly by ATM, which phosphorylates p53 directly and activates other kinases (e.g. Chk2). UV light activates a different set of enzymes, including ATR, HIPK2, and the p53 Ser 392 kinase, the subject of this thesis. PCAF and p300/CBP acetylate lysines on the C-terminus, and their activity is blocked by MDM2 (Adapted from ref. 2)

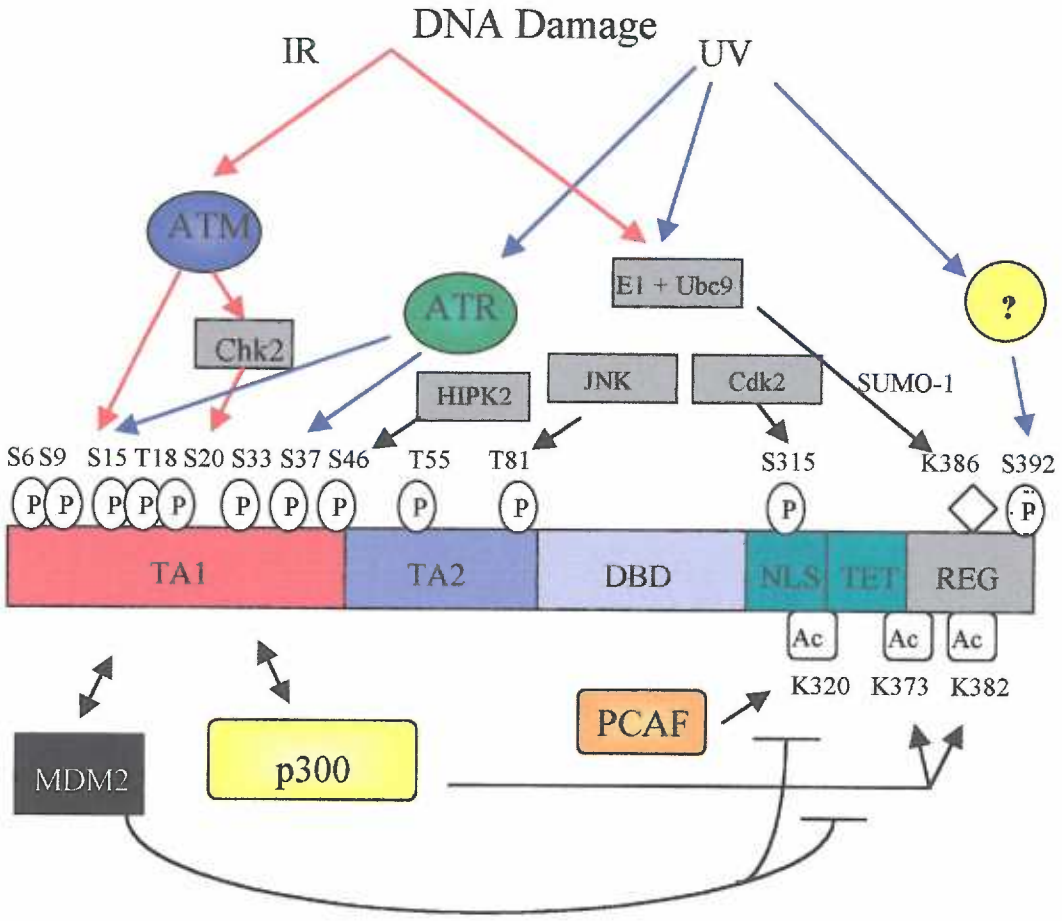
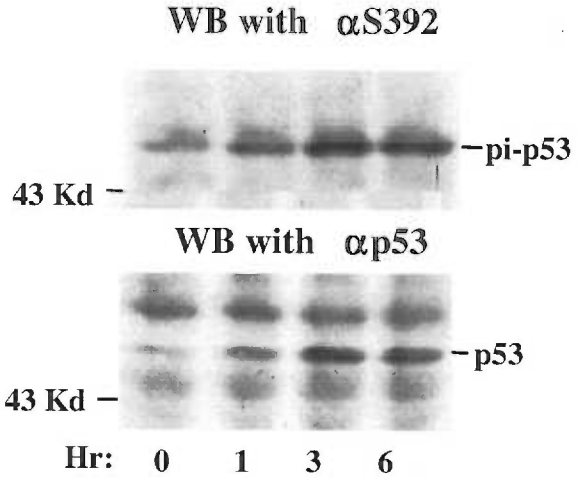

DNA Damage
IR
UV
ATM
ATR
Chk2
E1 + Ubc9
?
HIPK2
JNK
Cdk2
SUMO-1
S6 S9 S15 T18 S20 S33 S37 S46 T55 T81 S315 K386 S392
P
TA1
TA2
DBD
NLS
TET
REG
Ac
K320 K373 K382
MDM2
p300
PCAF

Figure 1.5. *Treatment of cells with cisplatin stablizes p53 and activates Ser 392 phosphorylation.* Murine testicular carcinoma F9 cells were treated with 20μM cisplatin and harvested at the indicated times. Cell lysates were run on SDS-PAGE followed by Western blotting analysis with antibodies against phosphorylated p53 Ser 392 (top panel) and against total p53 protein levels (bottom panel) using a polyclonal p53 antibody.


WB with αS392
pi-p53
43 Kd
WB with αp53
p53
43 Kd
Hr: 0 1 3 6

CHAPTER TWO

A DNA damage-induced p53 Serine 392 Kinase Complex Contains CK2, hSPT16, and SSRP1

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Running title: Identification and Purification of a p53 S392 Kinase.

Key words: p53 S392 kinase, DNA damage, FACT, SSRP1 and CK2.

(Note: This chapter appeared as Keller et al., (2001). Mol. Cell. 7: 283-292.)

SUMMARY

Phosphorylation of the human p53 protein at Ser 392 has been shown to be responsive uniquely to UV but not γ 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 (46, 96, 129). One group of stimuli includes genotoxic agents such as irradiation, chemicals, oxygen free radicals (38, 64, 105, 155). Others include withdrawal of growth factors (48), oncogenes (102, 144), hypoxia (49), and defects in nucleotide synthesis (98). As a consequence of these stresses, p53 is stabilized at the protein level and its transcriptional activity is enhanced (92). This activation of p53 leads, primarily through its transcriptional function (158), 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 post-translational modifications such as phosphorylation and acetylation (92, 139, 157). 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 (16). Probably the best characterized example of this is following ultraviolet (UV) or γ irradiation, which both cause DNA damage but activate distinct DNA repair pathways (97). After γ irradiation, several serine residues on both the N- and C-termini of p53 are targeted, possibly due to the action of the γ-responsive Ataxia Telangiectasia mutated (ATM) kinase (5, 164). This is strikingly demonstrated in A-T cells, which contain non-functional ATM, where p53 up-regulation is defective after γ irradiation (82, 86). Recent studies have shown that ATM may not directly phosphorylate p53, but instead may act through its downstream kinase, hChk2, which targets Ser 20 (8, 64). Phosphorylation at this site is known to impede binding of the p53 negative regulator, MDM2, thus increasing p53 stability (19, 157).

Phosphorylation of human 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 g irradiation (80, 105) and results in enhancement of sequence-specific binding activity *in vitro* (71), possibly by promoting stable tetramer formation (140). Substitution of Ser 392 with an acidic residue constitutively activates p53 as a transcription factor (56). The importance of Ser 392 in regulating p53 function is also reflected by the fact that this residue is evolutionarily conserved from humans through bony fishes (Figure 2.1). 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* (114). Recently, phosphorylation at this site *in vivo* was shown to be inhibited by p38MAPK-specific inhibitors (68, 83), suggesting that p38MAPK may target this serine directly or indirectly. Interestingly, p38MAPK was also reported to phosphorylate and activate CK2 after DNA damage (142), 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 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 7 polypeptides with a native molecular weight of ~700 Kd, 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) (131). This p53 Ser 392 kinase complex 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 g irradiation (80, 105). This appears to be a general phenomenon, as it occured 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 $20J/m^2$ UVC (105). Cells were treated with or without $20J/m^2$ UVC and harvested 8 hr later. Nuclear extracts were prepared and fractionated on a phosphocellulose (P11) column (Figure 2.2A). 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 (80). Kinase activity was detected in the 0.5M KCl fraction (top panel, lane 6) while endogenous p53

was detected largely in the 0.35M KCl fraction (lane 5) and residually in the 0.5 M KCl fraction (lane 6). Equal levels of the 311-393 peptides 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 2.2B (compare lane 5 with lane 6). Consistent with our hypothesis that UV stimulates p53 Ser 392 kinase activity, we detected faint phosphorylated endogenous p53 in the UV-treated fractions but not in the untreated (compare lane 3 with 4, and lane 5 with 6). CK2 alone was used as a positive control (lane 7). p38MAPK eluted in the 0.1M 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 0.5M 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 2.3A), and the fold purification was judged to be 40,900 (Table 2.1). 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 phosphospecific antibody (Figure 2.3B). 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 2.4). Kinase activity eluted at a native molecular weight of close to 670Kd (Figures 2.4A and B), suggesting that the kinase is part of a protein complex. Indeed, collodial blue staining analysis of the active proteins pooled from fractions 9 to 11 showed that this kinase complex is composed of at least 7 polypeptides (Figure 2.4C). 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 CK2α', p37 as CK2α, and p27 as CK2β. The identities of the other polypeptides are currently unknown due to the insufficient amounts of these large proteins 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 2.4) suggest that CK2 might associate with the FACT heterodimer that was recently identified as a transcriptional elongation factor (131). To test this, CK2, hSPT16, and SSRP1 were traced throughout a subsequent purification by WB using antibodies against these proteins (Figure 2.5). We observed that these proteins co-fractionated through all the columns used for purification (see Figure 2.3A for procedures). As shown in one representative WB result from a MonoQ column (Figure 2.5A), CK2α' and β subunits co-purified 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 2.4, CK2α' and p53 Ser 392 kinase activity were co-immunoprecipitated from the P11 0.5M KCl fraction by the affinity-purified polyclonal anti-hSPT16 and anti-SSRP1 antibodies, but not rabbit IgG (Figure 2.5B). 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 ~ 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 specificity

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-DMDM2, the p53 C-terminus (aa 311-393) or histone H1 (Figure 2.6A). 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 perferentially targeted the p53 fragment, with little activity on casein (compare lanes 2-3 with lanes 5-6). Also, this kinase complex did not phosphorylate GST-ΔMDM2 and histone H1 as efficiently as CK2 alone (compare lanes 8, 9, 11 and 12 with lanes 5-6). 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 actvitiy 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 2.6B, CK2 alone phosphorylated substrates 311-393 (C-terminal p53) and casein equally well (compare lanes 1 and 8). However, upon 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. 100ng 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 2.6C). Furthermore, the affect of hSPT16 and SSRP1 is not non-specific, because an N-terminal mutant of SSRP1 cannot reproduce the effect of the full-length protein (Figure 2.6C, 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 2.6B). In Figure 2.6C phosphorylation of SSRP1 was not visualized because less protein was added (25ng compared to 100ng in Figure 2.6B). 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 functions

Phosphorylation at Ser 392 is known to enhance sequence-specific DNA binding *in vitro* (71). To test the functional consequence of phosphorylation of p53 by the

purified kinase, gel mobility shift analysis was performed (Figure 2.7A and B). *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 p21wafl/cip1 promoter. Adding the purified kinase alone without ATP enhanced p53 binding (compare lane 2 with 3, 5, and 7 of Figure 2.7A), 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 (149). 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 2.7B, 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 2.7C). 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 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 4-5 fold increase were 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 2.7D). p53-null H1299 cells were transiently transfected with either WT or S389A mutant p53, as well as a p53RE-luciferase construct derived from the $p21^{WAF1/CIP1}$ promoter and β-galactosidase as an internal control. 48 hours after transfection, the cells were UV-treated and harvested one or two hours after treatment. As shown in the graph, p53-dependent luciferase activity increased 2-3 fold following UV irradiation in the cells ectopically expressing WT but not mutant p53. IP's 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 7 polypeptides with a native molecular mass of approximately 700 Kd. 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 co-fractionation of these proteins and by co-immunoprecipitation 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. This suggests a possible new mechanism that may regulate p53 functions 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 2.7A-C). 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 2.7D). 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 (18).

Because Ser 392 is functionally important, identification of the kinase that targets this site is crucial for understanding p53 biology. Two kinases, CK2 and p38MAPK, have been shown to phosphorylate Ser 392 *in vitro* (68, 83, 114). 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 (68, 83). However, there is no clear evidence demonstrating that this kinase is directly responsible for Ser 392 phosphorylation in response to DNA damage. Although it phosphorylates Ser 392 and enhances p53's sequence-specific DNA binding activity *in vitro* (71), 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 multi-subunit 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 (52), but not a p38MAPK site (125); 2) p53 Ser 392 kinase activity was barely detected in any other fractions other than the P11 0.5M fraction (Figure 2.2A), from which a kinase complex containing CK2 was purified (Figure 2.4). 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 (142). This may well explain the inhibition of Ser 392 phosphorylation by p38MAPK-specific inhibitors (68, 83). 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 2.6). Little has been done to show how CK2 is regulated *in vivo*, despite the fact that it has over 160 known targets (52). 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 the first 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 co-fractionated with CK2 throughout the purification (Figs. 2.4 and 5 and data not shown), and we further demonstrated the association by using antibodies against hSPT16 and SSRP1 to immunoprecipitate CK2α' and p53 Ser 392 kinase activity (Figure 2.5B). FACT activity was recently identified coincidently in the P11 0.5M KCl fraction from HeLa nuclear extracts, as a factor that enhanced the processivity of RNA polymerase II through a nucleosome array *in vitro* (130), and purified as a heterodimer with the human homologs of *Saccharomyces cerevisiae* Spt16 and yeast Pob3 (SSRP1) (131). 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 family of proteins (14), which are highly abundant non-histone chromatin proteins (50). Interestingly, SSRP1 was identified initially in a peptide library screen for human proteins that bound to DNA modified by the anticancer drug cisplatin (14). This

highlights the fact that SSRP1 has multiple functions, including DNA damage recognition and transcription elongation.

Identification of the hSPT16-SSRP1-CK2 complex raises an intriguing possibility 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 which 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 (31, 100, 101, 170). RNA polymerase II stalls when it encounters DNA lesions caused by agents such as UV and cisplatin, leading to a drop in mRNA synthesis (97). Accumulation and activation of p53 following UV and cisplatin in normal human fibroblasts was found to closely parallel suppression of mRNA synthesis (101). 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 (101), which suggests that the p53 Ser 392 kinase complex is activated specifically after UV, but not after γ 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 (101). Is actinomycin D, which intercalates into the DNA and induces p53 (81), also recognized by SSRP1 and hSPT16? Also, the serine/threonine kinase

inhibitor DRB is an effective agent against CK2 (174), and can also inhibit transcription initiation (33, 143) and/or elongation (22). However, DRB does not appear to inhibit CK2 phosphorylation of Ser 392, but rather activates and stabilizes p53 (10, 101)? 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 provide 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

Abbreviations. CK2, casein kinase 2; DTT, dithiothreitol; EMSA, electro-mobility shift assay; FACT, facilitates chromatin transcription; SSRP1, structure specific recognition protein; IP, immunoprecipitation; MDM2, a gene amplified in mouse double-minute chromosome; p53RE, p53 responsive DNA element; P11, phosphocellulose; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UV, ultraviolet; WB, western blot; WT, wild-type.

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 0.2mM PMSF, 4μM pepstatin A, 1μg/ml leupeptin, and 1μg/ml aprotinin. BC100 buffer was used for IP assays and included phosphatase inhibitors NaF (100μM) and Na-ortho-vanadate (100μM). 1x kinase buffer is 20mM TRIS/HCl (pH 7.5), 10mM $MgCl_2$,

and 1mM DTT.

Plasmids and antibodies. The his-p53 expression vector was obtained from Ze'ev Ronai (Mount Sinai Medical School, New York). pET-311-393 expression vector was generously provided by Nikola Pavletich (Memorial Sloan Kettering Cancer Center, New York). pET-mp53-S392A expression vector was as described (80). 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-CK2α' and polyclonal anti-p53 antibodies were from Santa Cruz Biotech., Inc, and anti-CK2β antibody was from Transduction Laboratory, Inc. Anti-Ser 392 phosphospecific p53 antibody was prepared as previously described (80, 145), as was PAb421 (58). 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 4.5g/L glucose and with L-glutamine (Gibco BRL), and supplemented with 10% fetal bovine serum (FBS), 50 units/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 wildtype 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_2 atmosphere.

Purification of recombinant proteins, affinity purification of antibodies, and purification of the recombinant p53 Ser 392 kinase complex. His-p53, his-SSRP1, and his-mSPT16 (aa 802-1047), and his-p63γ 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 (a.a.311-393) peptide was purified on an HS 4.6/100 column (BioCad Sprint HPLC) and protein was eluted in a 40ml linear gradient from 0.1M to 0.8M KCl (BC-100 to BC-800 buffer). 311-393 peptide eluted at the end of the gradient and was judged ~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 (57). The recombinant p53 Ser 392 kinase complex containing 5μg his-SSRP1, 10μg flag-hSPT16, and 1 unit of CK2 was purified on a Superdex 200 gel filtration column (Pharmacia).

Purification of p53 Ser 392 kinase. F9 cells were grown to 80% confluency on plates and then half the plates were treated with 20J/m^2 UVC (2x10^9 cells total). Treated cells were harvested 8 hours post-UV and both treated and untreated cells used for nuclear extract preparation as previously described (105). Nuclear extracts were loaded onto a phosphocellulose (P11) column and fractions were collected at 0.1M KCl (BC-100), 0.35M KCl (BC-350), 0.5M KCl (BC-500), and 1.0M 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 (24mg

protein) was loaded onto a DEAE sephacyl column (Pharmacia Biotech) and step-eluted as above. The BC-350 fraction (14mg) was further purified on an HQ 4.6/100 column (BioCad Sprint HPLC), and protein was eluted in a 30ml linear gradient from BC-100 to BC-800. Active fractions (4.1mg) were pooled and run on an HS 4.6/100 column, and eluted in the same manner. Active fractions (1.2 mgs) were again pooled and loaded onto a Heparin Hi-Trap column (1ml, Pharmacia). A 20ml linear gradient was run, again from BC-100 to BC-800 and active fractions (approx. 500μg) 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 micro-concentrator spin column (Amersham). 50μl 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 700kD 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, fifteen 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 (VDNIQAGELTEGIWRFDEISFVNFAR). 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: GSRARVYAEVNSLRSREYW; 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 (103) using [γ-^{32}P]-ATP. Substrates included 100ng of his-p53, 100ng of 311-393, 100ng histone H1, 200ng GDMDM2, or 1μg casein.. Either 3μl of the column fractions or 20ng of the purified p53 Ser 392 kinase, or 0.5 unit CK2 (Promega) was used. Kinase assays were also done using unlabeled ATP (1mM) followed by SDS-PAGE and then phosphorylated 311-393 was detected by WB using the anti-Ser 392 antibody.

Western blotting (WB), immunoprecipitation (IP), and IP-kinase assays. Protein samples were run on SDS-PAGE, followed by transfer to nitrocellulose membranes and which were then immunoblotted with the appropriate antibodies. Proteins were detected by ECL reagents (Santa Cruz Biotech). hSPT16, SSRP1, and CK2α' were immunoprecipitated from the P11 0.5M KCl fraction. Anti-hSPT16 or anti-hSSRP antibodies (1μg) were combined with 10μl Protein G Sepharose 4B fast-flow beads (Pharmacia) and 50μg protein for 2 hours at 4°C in a total volume of 400μl BC100 buffer. The IPs were washed 3 times in BC100 and one time in 1x 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 (EMSA), *in vitro* transcription assay, and luciferase assay. Kinase assays were performed as described above, except that reaction volumes were 10μl. Following this a DNA binding assay was performed as previously published (85, 103). 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, 0.1-1.0ng DNA/assay) derived from the p21$^{WAF1/CIP1}$ promoter (177) for 20min. at room temperature in a final volume of

20μl. Unlabeled oligomers (100ng) including poly(dIdC) and p53RE were used for competition studies. Antibodies (0.1μg) including the p53 antibody PAb 1801 and anti-CK2α' 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 (104, 106). The transient transfection and luciferase assays were done as described (178).

ACKNOWLEDGMENTS

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Figure 2.1. *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.

Ser 392 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 2.2. *p53 Ser 392 kinase activity is stimulated by UV.* **(A)** p53 Ser 392 kinase activity is present in the 0.5M KCl fraction. F9 cells were treated with $20J/m^2$ UVC for 8 hours, and harvested for nuclear extract preparation. Extracts (from 10^9 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 1μg of the indicated fraction and unlabeled ATP and 100ng p53 C-terminal peptide (a.a. 311-393) as substrates. Phosphorylated products were detected by WB with a phosphospecific 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 by pi-mp53. **(B)** p53 Ser 392 kinase activity in the P11 0.5M 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 mg protein) was performed identically as in panel A.

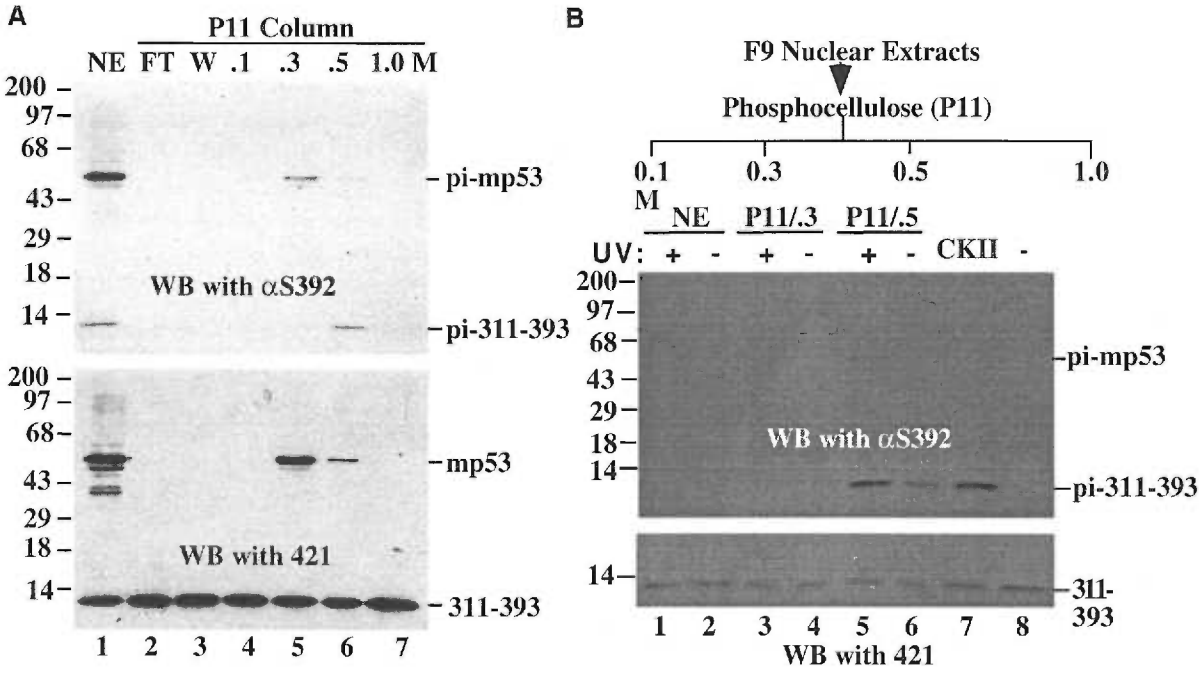
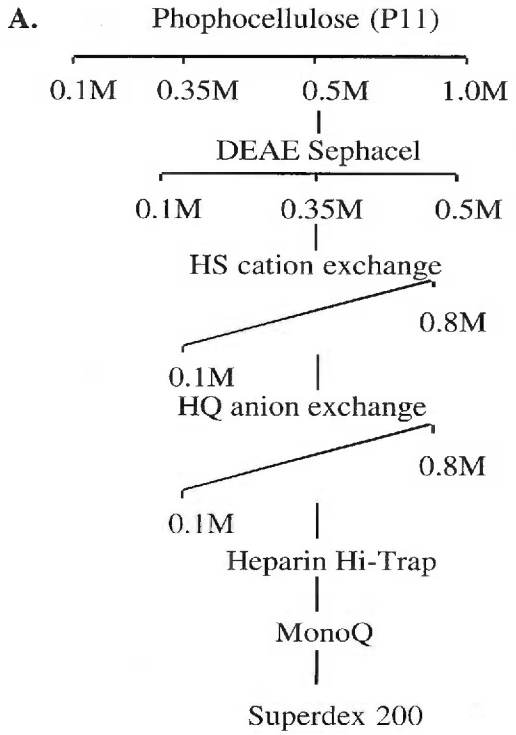

A
P11 Column
NE FT W .1 .3 .5 1.0 M
200
97
68
43
29
18
14
pi-mp53
WB with αS392
pi-311-393
mp53
WB with 421
311-393
1 2 3 4 5 6 7
B
F9 Nuclear Extracts
Phosphocellulose (P11)
0.1 M
0.3
0.5
1.0
NE
P11/.3
P11/.5
UV: + - + - + - CKII -
pi-mp53
WB with αS392
pi-311-393
311-393
1 2 3 4 5 6 7 8
WB with 421

Figure 2.3. *Purification scheme of the 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 2.1 for details. **(B)** Anti-Ser 392 antibodies selectively detected p53 Ser 392 kinase activity in the MonoQ fractions. Column fractions (3 μl) were used in a radioactive kinase assay (top panel) and a WB kinase assay using the anti-Ser 392 antibody (middle panel). 100ng C-terminal p53 311-393 detected by PAb421 was used as a substrate in each reaction (bottom panel).


A.
Phophocellulose (P11)
0.1M
0.35M
0.5M
1.0M
DEAE Sephacel
0.1M
0.35M
0.5M
HS cation exchange
0.8M
0.1M
HQ anion exchange
0.8M
0.1M
Heparin Hi-Trap
MonoQ
Superdex 200

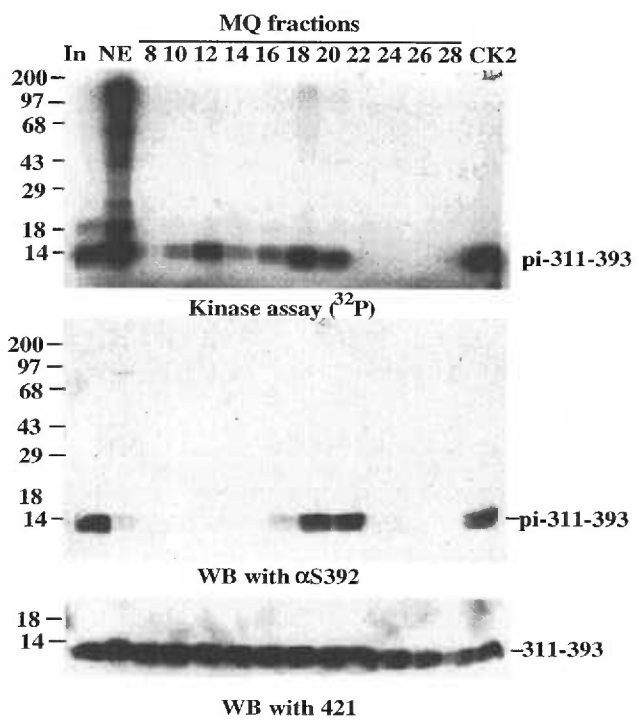

B.
MQ fractions
In NE 8 10 12 14 16 18 20 22 24 26 28 CK2
200
97
68
43
29
18
14
pi-311-393
Kinase assay (32P)
200
97
68
43
29
18
14
pi-311-393
WB with αS392
18
14
311-393
WB with 421

Figure 2.4. *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 700kD as judged by both a radioactive kinase assay using [γ^{32}P]ATP (top) and a WB kinase assay (middle). Column fractions (3 µl) were used in each lane with 100ng C-terminal p53 311-393 (detected by PAb 421 in bottom panel). **(C)** Fractions 9-11 (~7.5µg protein) were pooled and subjected to SDS-PAGE and stained with collodial blue, followed by digestion for mass spectrometry (MS). Corresponding proteins identified by MS analysis were labeled on the right of the panel. * Denotes the possible contaminating protein, as it did not show in a separate purification.

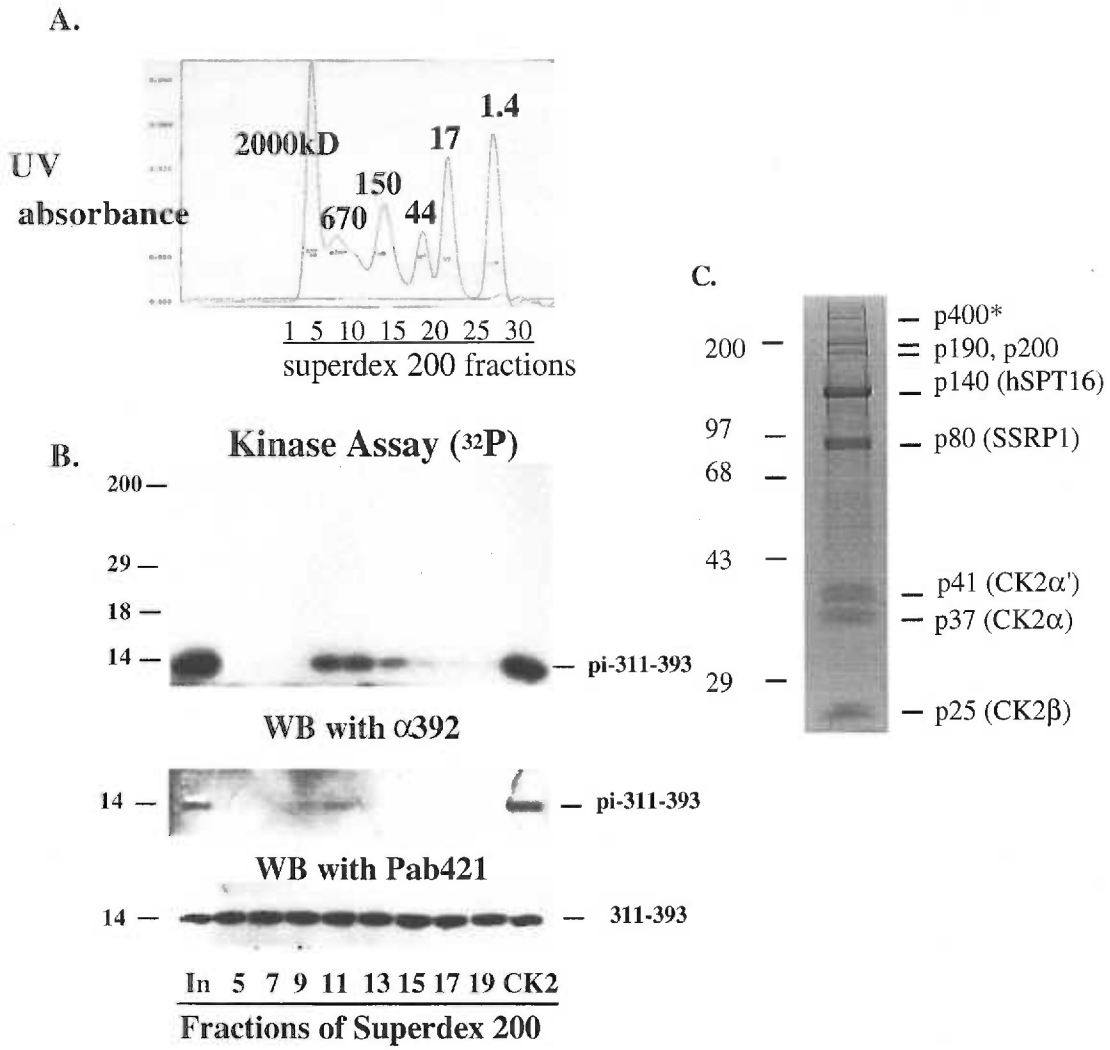

A.
UV
absorbance
2000kD
670
150
44
17
1.4
1 5 10 15 20 25 30
superdex 200 fractions
B.
Kinase Assay (32P)
200
29
18
14
pi-311-393
WB with α392
14
pi-311-393
WB with Pab421
14
311-393
In 5 7 9 11 13 15 17 19 CK2
Fractions of Superdex 200
C.
200
97
68
43
29
p400*
p190, p200
p140 (hSPT16)
p80 (SSRP1)
p41 (CK2α')
p37 (CK2α)
p25 (CK2β)

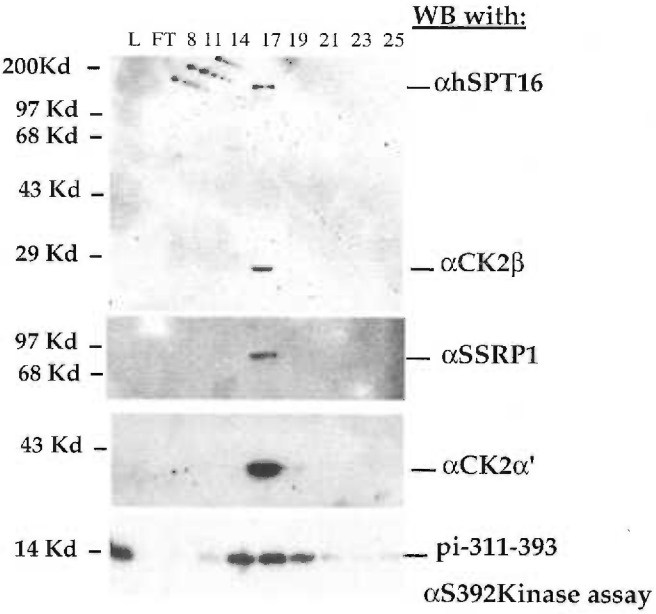
Table 2.1. *Fold purification of p53 Ser 392 kinase.* Fractions from purification steps were used in an *in vitro* kinase assay using the phosphospecific αSer 392 antibody to detect phosphorylated product. Following WB, the visualized bands were quantified by densitometry using BioRad Molecular Analyst software and listed in the Table as kinase units. Specific activity was measured by dividing the kinase units in each sample by the total amount of protein in that sample. Abbreviations: NE (nuclear extract), P11 0.5 (phosphocellulose 0.5 M KCl fraction), DE 0.35 (DEAE Sephacyl 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).

Table 1: Fold purification of Ser 392 kinase

Fraction	Protein (mgs)	Kinase units (Western)	Specific Activity (U/mg)	Fold purification
NE	430	6080	14	1
$P_{11}0.5$	24	30,700	1280	91
DE 0.35	14	87,600	6260	450
HQ Pool	4.1	129,000	31,500	2250
HS Pool	1.2	120,000	100,000	7140
S200 frac.10	0.0025	1430	572,000	40,900

Figure 2.5. *hSPT16 and SSRP1 associate with CK2.* **(A)** Representative column from the purification (MonoQ) showing co-elution of CK2α' and β subunits with hSPT16 and SSRP1. Column fractions (15μl) were run on SDS-PAGE for WB with the indicated antibodies. A WB kinase assay was performed as described above (bottom panel), and demonstrate that p53 Ser 392 kinase activity peak co-elutes with these proteins. L indicates the material loaded onto the column. **(B)**. IP of CK2 and p53 Ser 392 kinase activity with hSPT16 and SSRP1. IP's were done using either normal rabbit IgG, αhSPT16, or αSSRP1 antibodies (1μg 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 WB-kinase assay to pull down p53 Ser 392 kinase activity using 311-393 as a substrate. * Indicates IgG heavy chain.

A.


WB with:
L FT 8 11 14 17 19 21 23 25
200Kd
97 Kd
68 Kd
43 Kd
29 Kd
αhSPT16
αCK2β
97 Kd
68 Kd
αSSRP1
43 Kd
αCK2α'
14 Kd
pi-311-393
αS392Kinase assay

B.

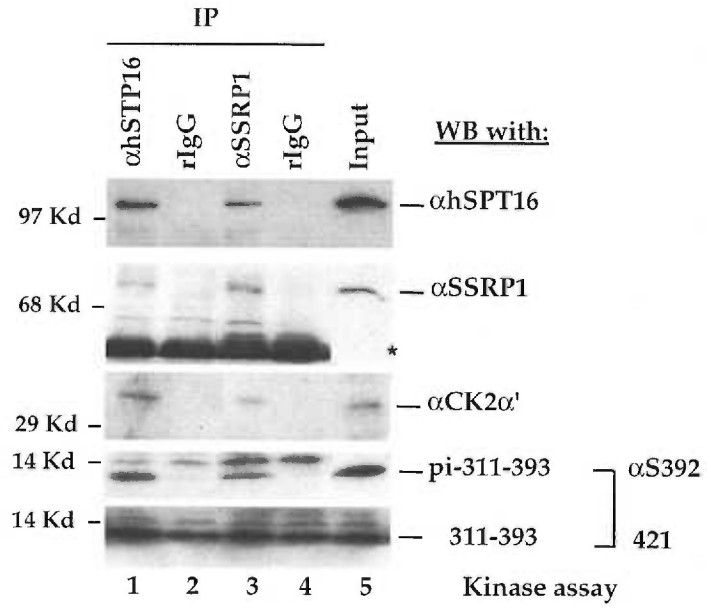

IP
αhSTP16
rIgG
αSSRP1
rIgG
Input
WB with:
97 Kd
αhSPT16
68 Kd
αSSRP1
*
29 Kd
αCK2α'
14 Kd
pi-311-393
αS392
14 Kd
311-393
421
1 2 3 4 5
Kinase assay

Figure 2.6. *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 (a.a.311-393, 100ng) equally well in an *in vitro* radioactive kinase assay, but p53 Ser 392 kinase does not efficiently phosphorylate casein (1μg), an N-terminal deleted, GST-fusion MDM2 (GMDM2, 50ng), or histone H1 (100ng). 0.5 unit of CK2 (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 100ng) and/or flag-hSPT16 (4 and 100ng). rhSPT16 and rSSRP1 indicate recombinant proteins **(C)** The substrate switching as seen above is not due to a non-specific protein-protein interaction because N-terminal SSRP1 (bp 1-719) does not affect casein phosphorylation. A radioactive kinase assay was done as above with 10ng N-SSRP1, 25ng his-SSRP1, 25ng flag-hSPT16, 100ng his-p53, and 1μg casein. * The mirgration of p53 in lanes 12-13 changed due to cleavage of the histidine tag by contaminating thrombin in the N-SSRP1 preparation.

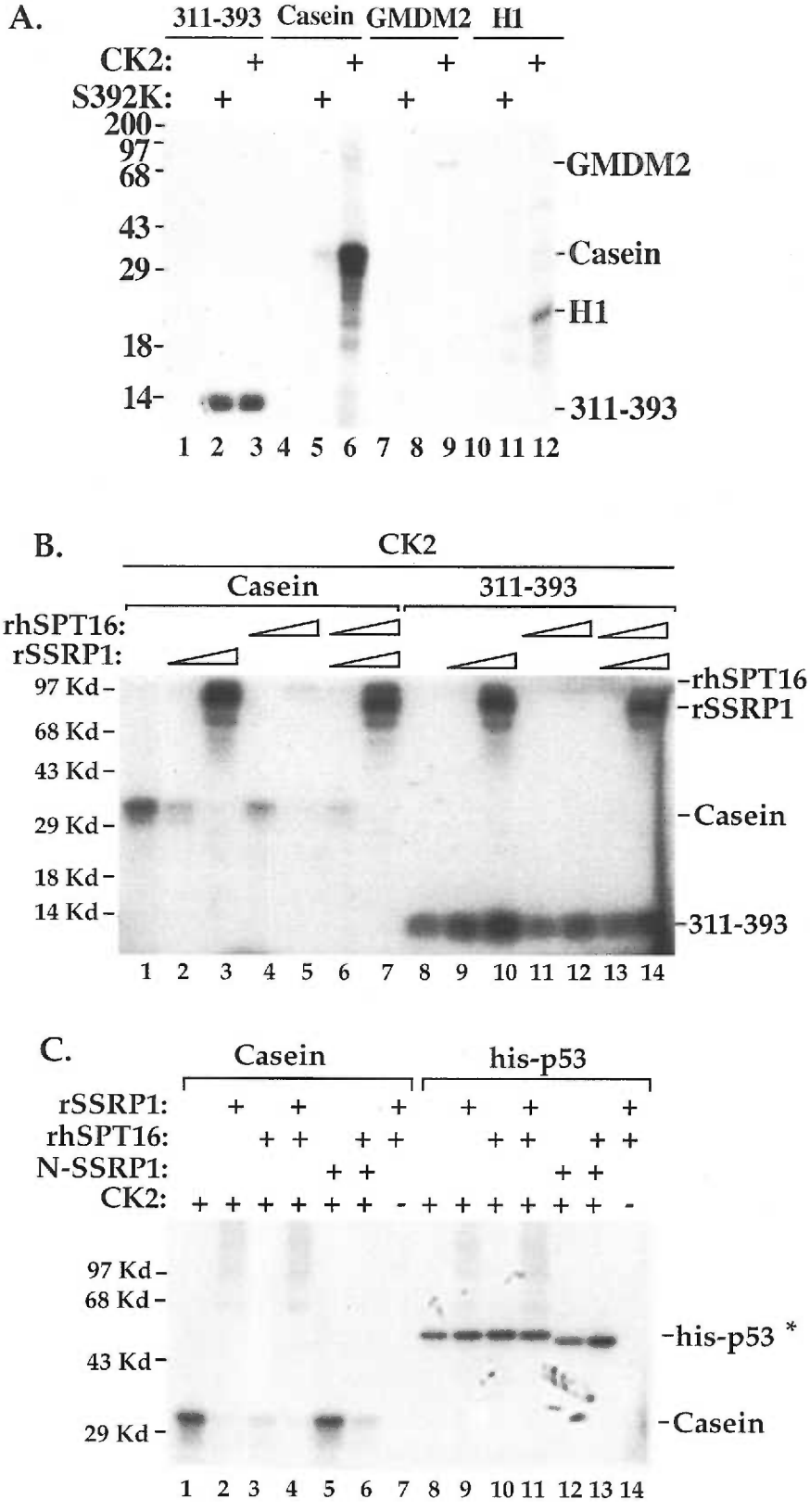
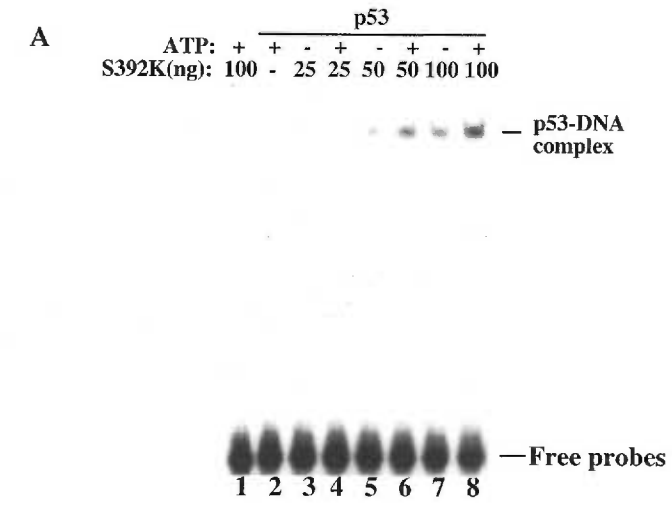
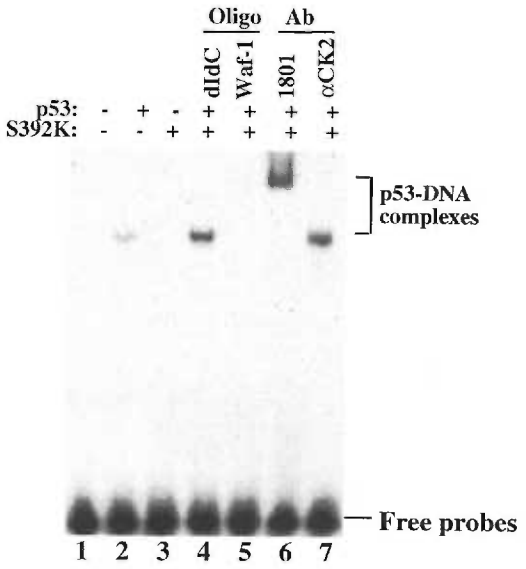
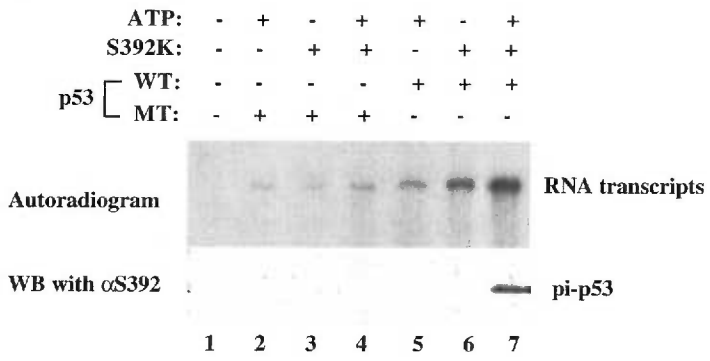

A.
311-393
Casein
GMDM2
H1
CK2:
S392K:
200-
97-
68-
43-
29-
18-
14-
-GMDM2
-Casein
-H1
-311-393
1 2 3 4 5 6 7 8 9 10 11 12
B.
CK2
Casein
311-393
rhSPT16:
rSSRP1:
97 Kd-
68 Kd-
43 Kd-
29 Kd-
18 Kd-
14 Kd-
-rhSPT16
-rSSRP1
-Casein
-311-393
1 2 3 4 5 6 7 8 9 10 11 12 13 14
C.
Casein
his-p53
rSSRP1:
rhSPT16:
N-SSRP1:
CK2:
97 Kd-
68 Kd-
43 Kd-
29 Kd-
-his-p53 *
-Casein
1 2 3 4 5 6 7 8 9 10 11 12 13 14

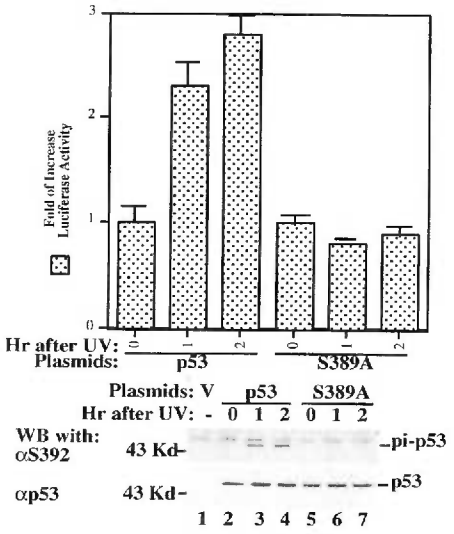
Figure 2.7. *The purified p53 Ser 392 kinase complex enhances p53's sequence-specific DNA binding and transcriptional activity in vitro.* **(A)** Cold kinase assays were performed, followed by an electro-mobility shift assay (EMSA) using full-length his-p53 (50ng) that was phosphorylated by increasing 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 p21waf1/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 panel A. Two unlabeled oligomers were used, poly(dIdC) (100ng, lane 4) and p53RE (100ng, lane 5). Also, antibodies (0.1μg each) were used which were specific against p53 (PAb 1801, lane 6) and CK2α' subunit (lane 7). **(C)** The p53 Ser 392 kinase stimulates transcription mediated by WT but not mutant (MT) p53. A reconstituted transcription system was used containing purified RNA pol II (50ng), general transcription factors (GTFs), and a DNA template containing a p53RE motif upstream from the TATA box derived from adenovirus major late promoter. 100ng WT or MT p53 and 4μl 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 WB 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, 6 well plate) were transfected with plasmids (1μg DNA total) encoding no gene, murine WT p53, or S389A mutant (400ng), together with a luciferase reporter plasmid (100ng) driven by the p53RE motif derived from the p21 promoter and a β-galactosidase reporter plasmid

(200ng) driven by the CMV promoter. 48 h post-transfection, cells were UV-treated for 0, 1, or 2 h and then harvested for luciferase and β-gal assays. Luciferase activity was normalized by the internal β-gal activity. Fold increase in luciferase activity was calculated and is presented in the graph (each column is from 3 independent assays, and bars show standard deviation). The bottom panels show p53 expression as detected by IP with PAb 421 and WB with either αSer 392 or αp53 polyclonal antibodies.


A
p53
ATP: + + - + - + - +
S392K(ng): 100 - 25 25 50 50 100 100
p53-DNA complex
Free probes
1 2 3 4 5 6 7 8


B
Oligo
Ab
dIdC
Waf-1
1801
αCK2
p53: - + - + + + +
S392K: - - + + + + +
p53-DNA complexes
Free probes
1 2 3 4 5 6 7


C
ATP: - + - + + - +
S392K: - - + + - + +
p53 WT: - - - - + + +
MT: - + + + - - -
Autoradiogram
RNA transcripts
WB with αS392
pi-p53
1 2 3 4 5 6 7


D
Fold of Increase Luciferase Activity
Hr after UV: 0 1 2 0 1 2
Plasmids: p53 S389A
Plasmids: V p53 S389A
Hr after UV: - 0 1 2 0 1 2
WB with: αS392
43 Kd-
-pi-p53
αp53
43 Kd-
-p53
1 2 3 4 5 6 7

CHAPTER THREE

p53 serine 392 phosphorylation increases after UV through induction of the assembly of the CK2-hSPT16-SSRP1 complex

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Running title: Characterization of the CK2-hSPT16-SSRP1 complex.

Key words: p53 S392 kinase, DNA damage, hSPT16, SSRP1 and CK2.

SUMMARY

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 CK2's conformation 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.

INTRODUCTION

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 (160). Cellular insults that activate p53 include DNA damaging agents such as radiation and chemical mutagens (64, 80, 105, 155), hypoxia (49), ribonucleotide depletion (98), oxygen free radicals (38), and cellular and viral oncogenes (102, 144). 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 (46, 160). Concurrently, p53 protein levels dramatically increase through relief of targeted degradation to the proteosome (61, 90) and also through increased translation of the p53 mRNA (40, 81, 119). Thus many stress-activated signaling pathways connect to p53, that in turn activate expression of

down-stream effector pathways (160).

One of the mechanisms by which stress signals are communicated to p53 is through posttranslational modifications (2). These modifications such as phosphorylation and acetylation are believed to control p53's protein stability and transcriptional activity by affecting protein-protein interactions and intramolecular conformational changes (46). For example, phosphorylation at several sites on the amino (N)-terminal domain in response to stress (5, 8, 17, 64, 145) prevent binding of the MDM2 oncoprotein, an E3 ubiquitin ligase that targets p53 for degradation by the proteosome pathway (42, 67, 117). On the carboxy (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* (71, 84), possibly by stabilizing p53 tetramerization (140). 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 wildtype p53 in human osteosarcoma SAOS2 cells, but instead impaired p53's ability to suppress ras-mediated transformation in rat embryonic fibroblasts (24). 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 (56). 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 (80, 105). Casein kinase 2 (CK2) was originally identified as the kinase that targets this site *in vitro* (114). 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 (84). Our results showed that indeed the kinase was CK2, but it eluted from gel filtration chromatography in a high molecular weight fraction corresponding to approximately 700kD. 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 (131). 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 CK2's kinase activity shows that binding of hSPT16 and SSRP1 to CK2 inhibits casein phosphorylation while having no effect upon p53 phosphorylation, and that hSPT16 and SSRP1 are apparently not binding to the substrate binding pocket of CK2 but instead are inhibiting casein phosphorylation in an indirect fashion. Further, 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.

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 approximately 700kD (84). Seven polypeptides co-eluted with the kinase activity, five of which were identified as either 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 (Figure 3.1). When CK2 is run on the column alone, it elutes at close to the predicted molecular weight of the heterotetramer, 140kD (top panel). Interestingly, when recombinant hSPT16 and SSRP1 are mixed with CK2 and then run on the column, CK2's p53 kinase activity shifts to a high molecular weight fraction, at approximately 670kD, and at a similar molecular weight to the native p53 Ser 392 kinase complex (middle panel). The bottom panel is a Western blot for hSPT16 and SSRP1 when combined with CK2. The combined molecular weights of the proteins in the FACT-CK2 complex is predicted to be 360kD, thus we speculate that the recombinant complex contains multiple copies of some or all the components.

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 (Figure 3.2A). We generated mutants for both the N-terminus and middle region of hSPT16 (Figure 3.2A) 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 (D. Reinberg, personal communication, and our observations). The Coomassie-stained SDS-polyacrylamide (SDS-PAGE) gel in Figure 3.2A shows that equal levels of the proteins were used, including a GST only (GST-0) control.

As shown in Figure 3.2B, flag-WT-hSPT16 binds to GST-WT-SSRP1 *in vitro*, and also to GST-N-SSRP1 (amino acids 1-242), though 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 (a.a 321-640) (Figure 3.2B, right panel, lane 6). CK2 binding to SSRP1 and hSPT16 was tested by GST-pulldown followed by a kinase assay. Figure 3.2C shows that CK2 binds to GST-WT-SSRP1 and to GST-C-SSRP1 (a.a. 471-709) (Figure 3.2C, left panel, compare lanes 2 and 5), and there is also diminished binding to GST-mid-SSRP1 (a.a. 235-475) (compare lanes 2 and 4). CK2 also binds hSPT16 directly *in vitro* via the N-terminus (a.a. 1-329) (Figure 3.2C, 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 is summarized in Figure 3.2D.

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 $\alpha\alpha'\beta_2$ (52). 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 (Figure 3.2C). We tested the catalytic α' subunit and found that it binds to both GST-

WT-SSRP1 and GST-N-hSPT16 (Figure 3.3A, compare lanes 5 and 6), whereas the regulatory β subunit apparently binds much stronger to GST-WT-SSRP1 (Figure 3.3A, compare lanes 8 and 9). A Coomassie-stained gel is shown of the purified his-tagged CK2 proteins (Figure 3.3B).

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 pulldown assay exactly. That is, flag-N-SSRP1 bound exclusively to endogenous hSPT16 (Figure 3.3C, top panel, lane 2) and flag-C-SSRP1 bound exclusively to endogenous CK2α' subunit and p53 Ser 392 kinase activity (Figure 3.3C, bottom two panels, lane 3). Therefore, this cellular data confirms the SSRP1 interactions with hSPT16 and CK2 that were observed in the *in vitro* GST pulldown assay. Interestingly, though recombinant GST-C-SSRP1 migrates on SDS-PAGE faster than GST-N-SSRP1 (Figure 3.2A, compare lanes c and e), flag-C-SSRP1 stably expressed in RKO cells migrates slower than flag-N-SSRP1 (Figure 3.3C, compare lanes 2 and 3). One possibility for flag-C-SSRP1's slower migration is that the C-terminus of SSRP1 is highly modified in cells by posttranslational 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 Figure 3.4).

Based upon our protein-protein interaction experiments, we can present a model for the binding of hSPT16 and SSRP1 to the CK2 heterotetramer (Figure 3.3D). The CK2 crystal structure is solved (124) and resembles a butterfly, with the two regulatory β subunits making contacts along a two-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 580kD, close to the 670kD 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 CK2's kinase activity such that it phosphorylated p53 but inhibited its activity towards other substrates such as casein, histone H1, and MDM2 (84). Here we identify the p53 family member p63γ as an *in vitro* substrate for CK2 (Figure 3.4A, lane 4), though p63γ and p53 do not share sequence conservation in the C-terminal domain and there is no p63γ equivalent of Ser 392. We also tested p73α, another p53 family member, but found that CK2 does not phosphorylate this protein (Figure 3.4A, lane 7-9). Surprisingly, hSPT16 and SSRP1 inhibit the CK2-induced phosphorylation of p63γ (compare lane 4 with 5-6), 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 CK2's kinase activity 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 Figure 3.4C, and as seen previously (84), casein phosphorylation was strongly inhibited by his-WT-SSRP1 (compare lanes 1 and 2), though p53 phosphorylation was not affected (Figure 3.4, top panel, compare lanes 1 and 2). N-SSRP1, which does not bind CK2, also did not affect

CK2 activity towards casein or p53 (compare lanes 3 and 4). In contrast, both mid-SSRP1 and C-SSRP1 inhibited casein phosphorylation (compare lanes 5-8), though mid-SSRP1 inhibited to a greater degree. However, this result was surprising because C-terminal SSRP1 bound more strongly to CK2 (Figure 3.2C). As seen previously, casein phophorylation by CK2 was inhibited by flag-WT-SPT16 (Figure 3.4C, bottom panel, lane 9) though the truncation mutants appeared to inhibit only slightly (lanes 10-13). This data provides 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 (84). Our current data shows 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 (Figure 3.4C, top panel, lanes 4-8). Though 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 (127, 131, 165), or in its ability to bind to damaged DNA (14).

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 (Figure 3.5) (see Experimental procedures for details). The kinetic parameters show that when casein is used as the substrate, FACT induces a two-fold decrease in the maximal reaction velocity (V_{max}) and a 7-8 fold increase in the Michaelis-Menten constant (K_m), a measure of the efficiency of substrate utilization (Figure 3.5A). The efficiency of the enzymatic reaction (V/K) is also severely affected (~15 fold decrease) (Figure 3.5A). In contrast, p53 phosphorylation by CK2 is not affected by addition of FACT (Figure 3.5B), clearly demonstrating that FACT selectively modulates CK2's kinase activity by inhibiting its activity against other substrates. The 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 (80, 105). In our original purification of the p53 Ser 392 kinase we observed UV-responsive kinase activity in the phosphocellulose (P11) 0.5M KCl fraction, and used this fraction to purify the SSRP1-hSPT16-CK2 complex (Figure 3.6A) (84). In order to understand how the P11 0.5M 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 CK2α'. Surprisingly, when both non-treated and UV-treated F9 cell nuclear extracts were run on the P11 column and fractionated, hSPT16, SSRP1, and CK2α' protein levels all were dramatically increased in the UV-treated 0.5M KCl fraction (Figure 3.6B, 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, as 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 αSer 392 antibody (Figure 3.6B, bottom panel). First, the p53 Ser 392 kinase activity is increased in the nuclear extract after UV irradiation approximately three-fold as measured by densitometry. Second, the activity in the 0.5M 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 αSSRP1 antibody shows that SSRP1, hSPT16, and CK2α', and p53 Ser 392 kinase activity are associated in the 0.5M KCl fraction after UV treatment (Figure 3.6C). Because the P11 0.5M 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 (84). There is little known concerning the biochemical properties of the hSPT16-SSRP1 heterodimer, and few reports have identified other interacting partner proteins (37, 77, 79, 165). 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 (13, 127, 131), and is important for transcription and replication through chromatin. The biochemical role for this heterodimer (called FACT in mammalian cells) is not clear, though it may involve binding to histones H2A and H2B thereby disrupting the nucleosome octomer (131), and/or physically manipulating DNA by inducing negative supercoils (127). 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 (Figures 3.2 and 3.3C). Using GST-fusion protein binding assays we find that hSPT16 binds to the N-terminus of SSRP1, and SSRP1 binds to the central region of hSPT16 (Figure 3.2B and D). In cells, we have confirmed the N-SSRP1 and hSPT16 interaction by co-IP (Figure 3.3C, top panel). Interestingly, Brewster *et al.* (13) demonstrated in *S. 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.

Though 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 (165), the histone acetyltransferase complex NuA3 (77), and have independently have been found to elute from a size exclusion column at approximately 400kD (13), larger than the predicted size of 180kD for the yeast proteins. In mammals, an interaction has

been described between these proteins and the transcription initiation factor TFIIE (79). Here we show that the molecular weight of the recombinant CK2-FACT complex is approximately 700kD, the same size as the purified native complex (Figure 3.1) (84). This suggests that there are multiple copies of some or all of the proteins in the complex. For example, in Figure 3.3D we present a model in which there are two FACT heterodimers for every CK2 heterotetramer, which would make the predicted size of the complex 580kD.

In this study 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 (Figure 3.2C and D). In a similar experiment, CK2α' subunit binds to both hSPT16 and SSRP1 and CK2β subunit binds strongly only to SSRP1 (Figure 3.3A). Likewise, in cells, CK2 binds to the C-terminus of SSRP1 (Figure 3.3C). 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) (Figure 3.5A). In contrast, p53 phosphorylation at Ser 392 is not affected (Figure 3.5B). The drop in V_{max} indicates that hSPT16 and SSRP1 are binding to a site on CK2 that does not overlap the substrate binding pocket, and the increase in K_m indicates that casein no longers 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 p63γ, whose phosphorylation by CK2 is inhibited by SSRP1 and hSPT16 (Figure 3.4A). Also, based on our mutational analysis, the C-terminal two-thirds of SSRP1 is necessary for influencing CK2's substrate specificity (Figure 3.4C, bottom panel, lanes 5-8), and though full-length hSPT16 can also affect the substrate specificity (Figure 3.4C, 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.

Though Ser 392 was one of the first identified phosphorylation sites on p53 in cells (141), 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 (71). Indeed, several experimental approaches in cells and in mice have supported this notion (24, 56, 70, 84), 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 (80, 105). Thus it is of particular interest to identify the kinase that targets this site *in vivo* after UV irradiation.

Here we report that phosphorylation of p53 Ser 392 increases approximately three-fold in F9 cells following UV irradiation as measured by an *in vitro* WB-kinase

assay (Figure 3.6B, bottom panel). This UV-responsive kinase activity fractionated from the phosphocellulose column at 0.5M KCl (Figure 3.6A and bottom panel of 3.6B), and by WB analysis we detected dramatically increased protein levels of CK2, hSPT16, and SSRP1 (Figure 3.6B-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 (Figure 3.6B) 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, as elevating the SSRP1 level alone did not affect p53 Ser 392 phosphorylation (Zeng, S., Keller, D., and Lu, H., unpublished observations) nor did it affect p53 activity (175).

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 (Figure 3.6D). First, binding of FACT to CK2 *in vitro* is not sufficient to increase the specific activity of CK2 towards p53 (Figure 3.5B), 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 (Figure 3.5A) 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 (52). 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 (Figure 3.6B).

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 this proteins together. This hypothesis is based on the fact that SSRP1 is an HMG-box containing protein that preferentially recognizes cisplatin-modified DNA (14). Furthermore, Yarnell *et al.* (171) used *in vitro* gel-mobility shift assays to show that SSRP1's ability 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, though 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-hSPT16-SSRP1 interaction has recently been found in *S. cerevisiae*, using tandem-affinity purification (TAP) and mass spectrometry in a large-scale mapping of the yeast proteome (45). This finding implies that this complex may target non-p53 substrates, as there is no yeast p53 gene, and also that hSPT16 and SSRP1 may themselves be regulated by CK2.

EXPERIMENTAL PROCEDURES

Abbreviations. 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 gene amplified in mouse double-minute chromosome; P11, phosphocellulose; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UV, ultraviolet; WB, western blot; WT, wild-type.

Reagents and Buffers. Casein was purchased from Sigma. CK2 was purchased from Promega. Baculovirus expressing flag-hSPT16 was as described (84). 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 0.2mM PMSF, 4μM pepstatin A, 1μg/ml leupeptin, and 1μg/ml aprotinin. BC100 buffer was used for IP assays and included phosphatase inhibitors NaF (100μM) and Na-ortho-vanadate (100μM). 1x kinase buffer is 20mM Tris/HCl (pH 7.5), 10mM $MgCl_2$, and 1mM DTT. Lysis buffer consists of 50mM Tris/HCl (pH 8.0), 0.5% NP-40, 1mM EDTA, 150mM NaCl, 1mM DTT and protease inhibitors as above. Radioimmunoprecipitation asay (RIPA) buffer is 50mM Tris/HCl (pH 7.4), 150mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1mM DTT, and protease inhibitors as above.

Plasmids and antibodies. The his-p53 and pET-311-393 expression vectors were as described (84). The pRc/CMV/CK2α'-HA and pRc/CMV/CK2β-myc were generous gifts from David Litchfield (159) and the CK2-encoding cDNAs were subcloned into pET24a expression vectors (Novagen). pET28-antisense hSPT16 was from Danny Reinberg (UMDNJ, Robert Wood Johnson Medical School, NJ), and was used in PCR to generate N-terminal (a.a. 1-329) and mid-hSPT16 (a.a. 321-640) followed by subcloning into pET24a and pGEX-KG (Pharmacia) expression vectors. pKK233-3+SSRP1 plasmid was as described (84) and SSRP1 was then subcloned into pET24a and pGEX-KG expression vectors. N-terminal SSRP1 (a.a. 1-242), mid-SSRP1

(a.a. 235-475), and C-SSRP1 (a.a. 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-CK2α' and anti-p53 antibodies were from Santa Cruz Biotech., Inc, monoclonal anti-CK2β antibody was from Transduction Laboratory, Inc., and monoclonal anti-flag and anti-γ-tubulin antibodies were from Sigma. Anti-Ser 392 phosphospecific p53 antibody was prepared as previously described (80, 145), as was PAb421 (58). 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 (a.a. 321-640).

Purification of recombinant proteins and affinity purification of antibodies. Histidine-tagged proteins were purified on Ni-NTA 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 (Pharmacia) to remove the GST tag. The anti-SSRP1 antibody was purified as described previously (84).

Cell culture. Murine embryonic testicular carcinoma F9 cells containing wildtype p53 were grown on plates in Dulbecco's modified Eagle medium (DMEM) with 4.5g/L glucose and with L-glutamine (Gibco BRL), supplemented with 5% fetal bovine serum (FBS) and with penicillin/streptomycin. Human colorectal carcinoma RKO cells were grown on plates in DMEM supplemented with 10% FBS. All cells were grown at 37°C in a 5% CO_2 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. 1μg of fusion proteins were combined with 1μg of soluble his-WT-SSRP1, flag-WT-hSPT16, his-CK2α', or his-CK2β 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 RIPA buffer, and once with lysis buffer. They were then run on SDS-PAGE and transferred to PVDF membrane for Western blotting (WB). GST-pulldown 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 1x kinase buffer. WB-kinase reactions were carried out as previously described (84, 103) using ATP and 100ng his-p53 as substrates for 30min at 30°C.

Generation of flag-N- and C-SSRP1 cell lines. RKO cells were transfected with 3μg of either pCDNA3.1, pCDNA3.1 flag-N-SSRP1, or pCDNA-flag-C-SSRP1 expression constructs using Lipofectamine reagent (Invitrogen). 24h post-transfection, cells were trypsinized and transferred to 10cm plates at low density. 0.5mg/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 (84). The WB in Figure 3.6B was analyzed by a BioRad 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 CK2α' were

immunoprecipitated with anti-SSRP1 from the P11 0.5M KCl fractions of the F9 cell nuclear extract preparations as previously described. Radioactive *in vitro* kinase assays were performed with [γ-^{32}P]-ATP, in which the total ATP concentration (cold + hot) was 40μM. Substrates were either 100ng his-p53 or 1μg casein. In Figure 3.4C, 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 (1mM) followed by SDS-PAGE and then phosphorylated his-p53 was detected by WB using the anti-Ser 392 antibody.

Kinetic analysis. CK2 (0.5 units) was incubated with or without 4 pmol FACT (2 pmol flag-hSPT16 + 2 pmol his-SSRP1) on ice for 1 hr. *In vitro* radioactive kinase assays were then carried out in the presence of 250μM ATP (including 375μCi [γ-^{32}P]ATP) for a half hour while titrating substrates casein and his-p53. Casein concentrations ranging from 0.25-64μM and his-p53 ranging from 0.037-9.4μM were titrated into the CK2-FACT-ATP mixture. Reactions were analyzed by SDS-PAGE and the bands were cut out and radioactivity quantified using a Beckman model LS 6500 scintillation counter. Reaction velocities were obtained by measuring pmol 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.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. David Litchfield and Danny Reinberg for sharing plasmid reagents. We would also like to thank Hunjoo Lee for subcloning CK2α' and β cDNAs into histidine-tagged expression vectors and for expressing the recombinant proteins in *E. coli* cells, and Shelya Zeng for techinal assistance. Additionally, Drs. Michael Jackson and Lousie Rafty were particularly helpful by reviewing the kinetics data, as was Dr. John Denu for critically reviewing the manuscript. D. Keller is supported by the NEI predoctoral fellowship. This study is supported by NIH grants (CA095441 and CA93614) to H. Lu.

Figure 3.1. *The recombinant CK2-FACT complex is the same apparent molecular weight as the purified native one.* CK2 (Promega, 0.7pmol) was run on a superdex 200 column and fractions were analyzed in an αSer 392 WB kinase assay as described in Experimental Procedures (top panel). Next, recombinant flag-hSPT16 (rhSPT16, 20pmol) and his-SSRP1, (rSSRP1, 20pmol) were incubated with CK2 on ice for 1h followed by running on superdex 200 and analysis by kinase assay (middle panel). The FACT heterdimer 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*.

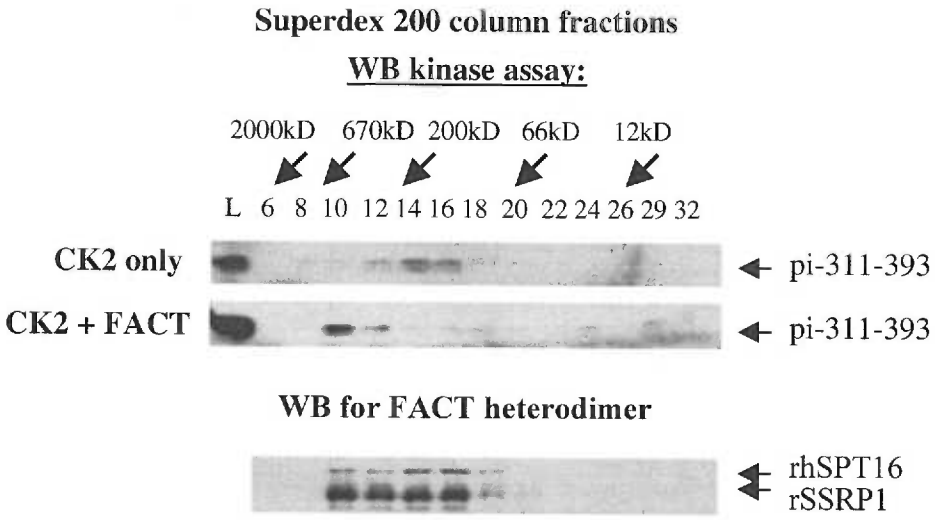
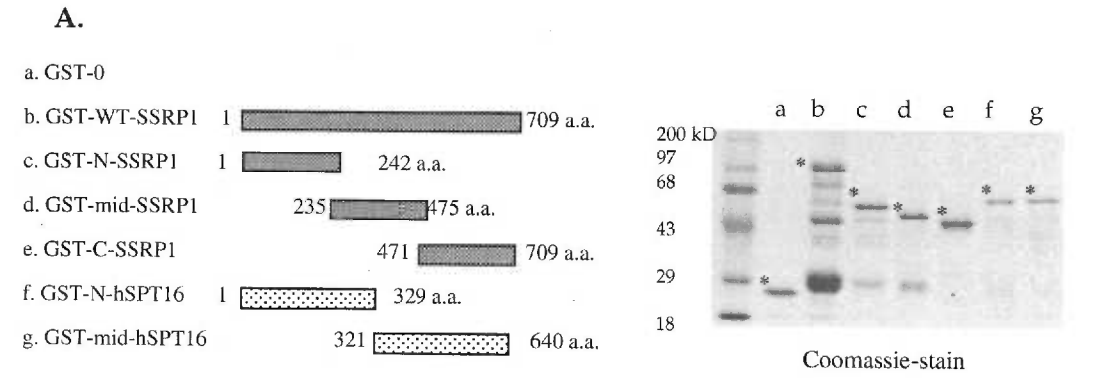
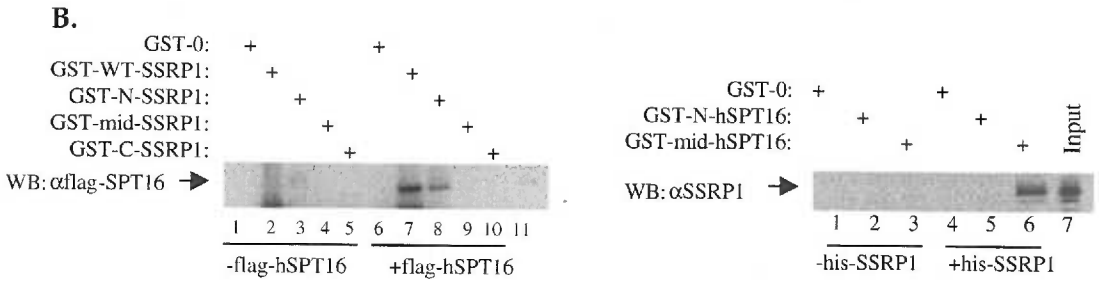
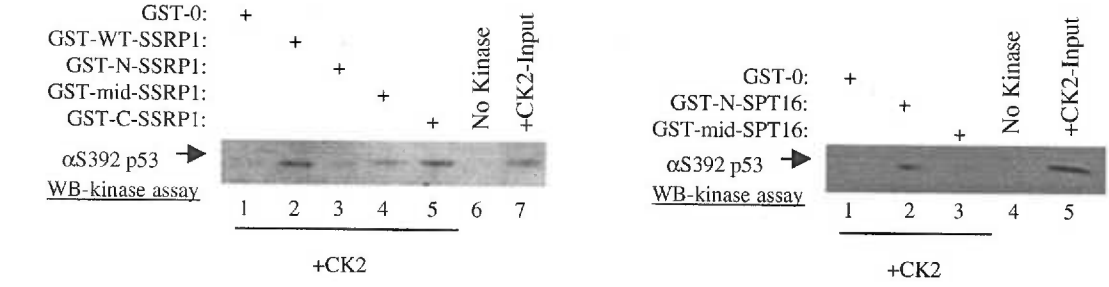

Superdex 200 column fractions
WB kinase assay:
2000kD 670kD 200kD 66kD 12kD
L 6 8 10 12 14 16 18 20 22 24 26 29 32
CK2 only
pi-311-393
CK2 + FACT
pi-311-393
WB for FACT heterodimer
rhSPT16
rSSRP1

Figure 3.2. *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 (1μg), immobilized on glutathione-agarose, were incubated either with 1μg 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 (1μg) were incubated with or without 1μg 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 100ng his-p53 as a substrate. **(D)** Schematic of the CK2-hSPT16-SSRP1 protein-protein interactions, based upon the above GST-pulldown experiments.


A.
a. GST-0
b. GST-WT-SSRP1 1 709 a.a.
c. GST-N-SSRP1 1 242 a.a.
d. GST-mid-SSRP1 235 475 a.a.
e. GST-C-SSRP1 471 709 a.a.
f. GST-N-hSPT16 1 329 a.a.
g. GST-mid-hSPT16 321 640 a.a.
a b c d e f g
200 kD
97
68
43
29
18
Coomassie-stain


B.
GST-0:
GST-WT-SSRP1:
GST-N-SSRP1:
GST-mid-SSRP1:
GST-C-SSRP1:
WB: αflag-SPT16
1 2 3 4 5 6 7 8 9 10 11
-flag-hSPT16
+flag-hSPT16
GST-0:
GST-N-hSPT16:
GST-mid-hSPT16:
Input
WB: αSSRP1
1 2 3 4 5 6 7
-his-SSRP1
+his-SSRP1


C.
GST-0:
GST-WT-SSRP1:
GST-N-SSRP1:
GST-mid-SSRP1:
GST-C-SSRP1:
No Kinase
+CK2-Input
αS392 p53
WB-kinase assay
1 2 3 4 5 6 7
+CK2
GST-0:
GST-N-SPT16:
GST-mid-SPT16:
No Kinase
+CK2-Input
αS392 p53
WB-kinase assay
1 2 3 4 5
+CK2

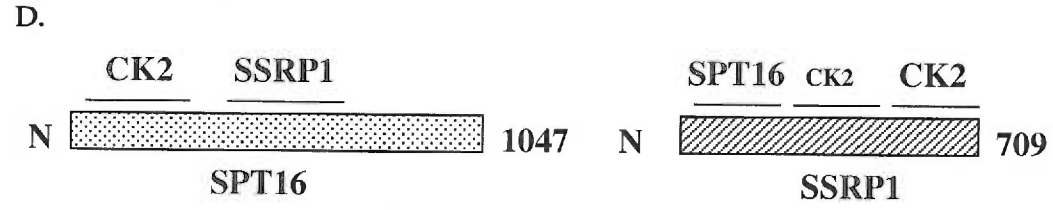

D.
CK2
SSRP1
N
1047
SPT16
SPT16
CK2
CK2
N
709
SSRP1

Figure 3.3. *Characterization of the hSPT16-SSRP1-CK2 complex.* **(A)** Mapping the CK2 subunit interaction with hSPT16 and SSRP1. CK2α' binds to both SSRP1 and hSPT16 while CK2β apparently binds stronger to SSRP1. GST fusion protein binding assays were done as above using 1μg of either CK2α' or β. 10% of the input was loaded directly onto the gel (lanes 10 and 11). **(B)** A Coomassie-stained gel showing the purified his-CK2α' 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 either 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 CK2α' (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 a.a. 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 weight of the recombinant complex *in vitro* is near 700kD (Figure 3.1).

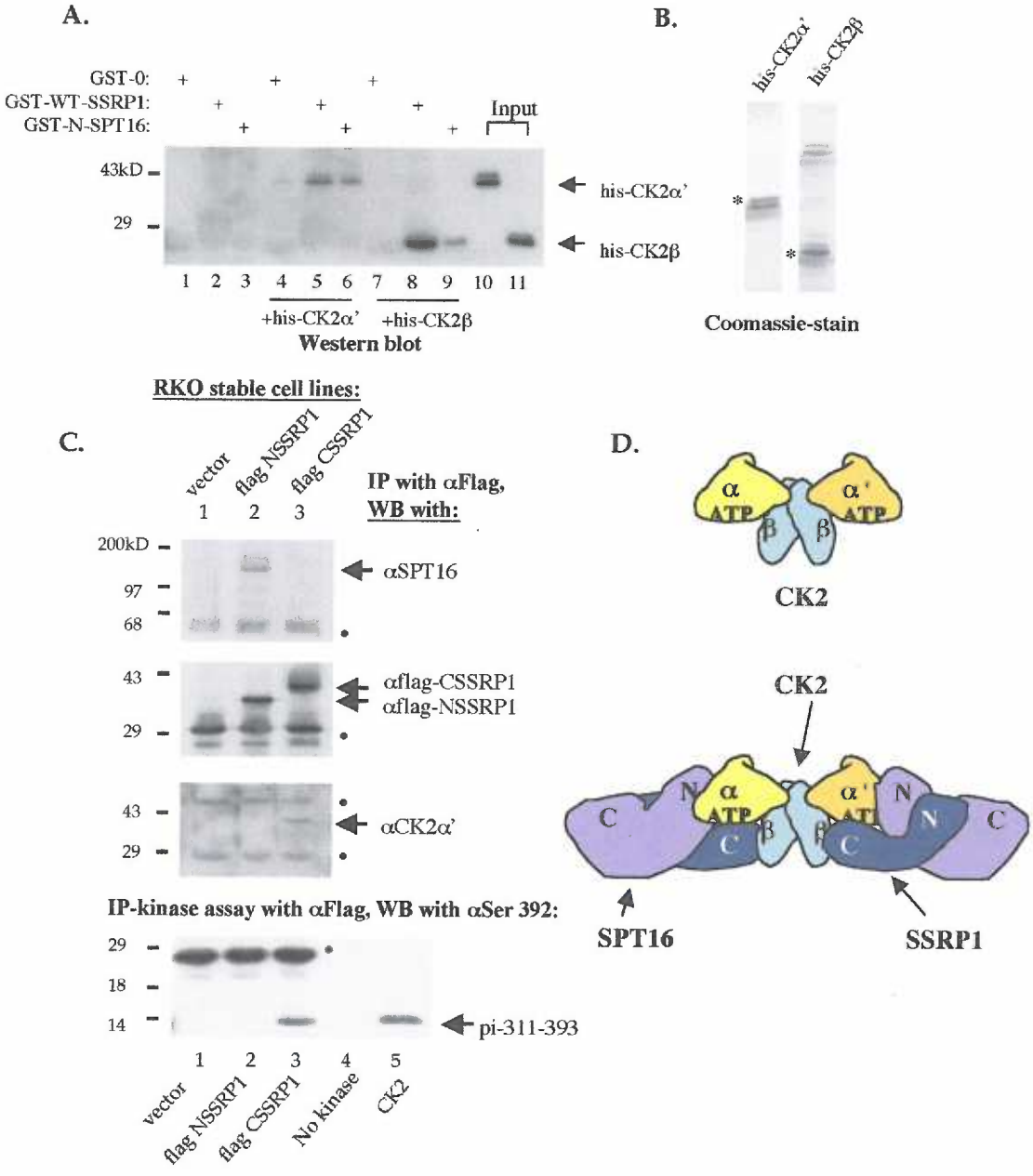

A.
GST-0:
GST-WT-SSRP1:
GST-N-SPT16:
Input
43kD
29
his-CK2α'
his-CK2β
1 2 3 4 5 6 7 8 9 10 11
+his-CK2α'
+his-CK2β
Western blot
B.
his-CK2α'
his-CK2β
Coomassie-stain
RKO stable cell lines:
C.
vector
flag NSSRP1
flag CSSRP1
1 2 3
IP with αFlag,
WB with:
200kD
97
68
αSPT16
43
29
αflag-CSSRP1
αflag-NSSRP1
43
29
αCK2α'
IP-kinase assay with αFlag, WB with αSer 392:
29
18
14
pi-311-393
1 2 3 4 5
vector
flag NSSRP1
flag CSSRP1
No kinase
CK2
D.
α ATP
α' ATP
β
β'
CK2
CK2
N
C
SPT16
SSRP1

Figure 3.4. *SSRP1 and hSPT16 influence the substrate specificity of CK2.* **(A)** The p53 family member p63γ, but not p73α, is phosphorylated by CK2, though 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 50ng his-p53, 150ng his-p63γ, or 500ng his-p73α as substrates. rFACT indicates recombinant SSRP1 and hSPT16 incubated together, titrated at 15ng and 30ng total protein. **(B)** Coomassie-stained SDS-PAGE of the substrates used in panel A (approximately 1μg of each protein loaded). The asterisks indicate the proteins, and his-p73α exists as two polypeptides. **(C)** A radioactive kinase assay was done as above with either his-p53 (100ng, top panel) or casein (1μg, bottom panel). CK2 was incubated along with the various SSRP1 and hSPT16 proteins as described in 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, though casein phosphorylation in lane 6 is inhibited, the signal is actually due to mid-SSRP1 (see lane 6, compare the top and bottom panels).

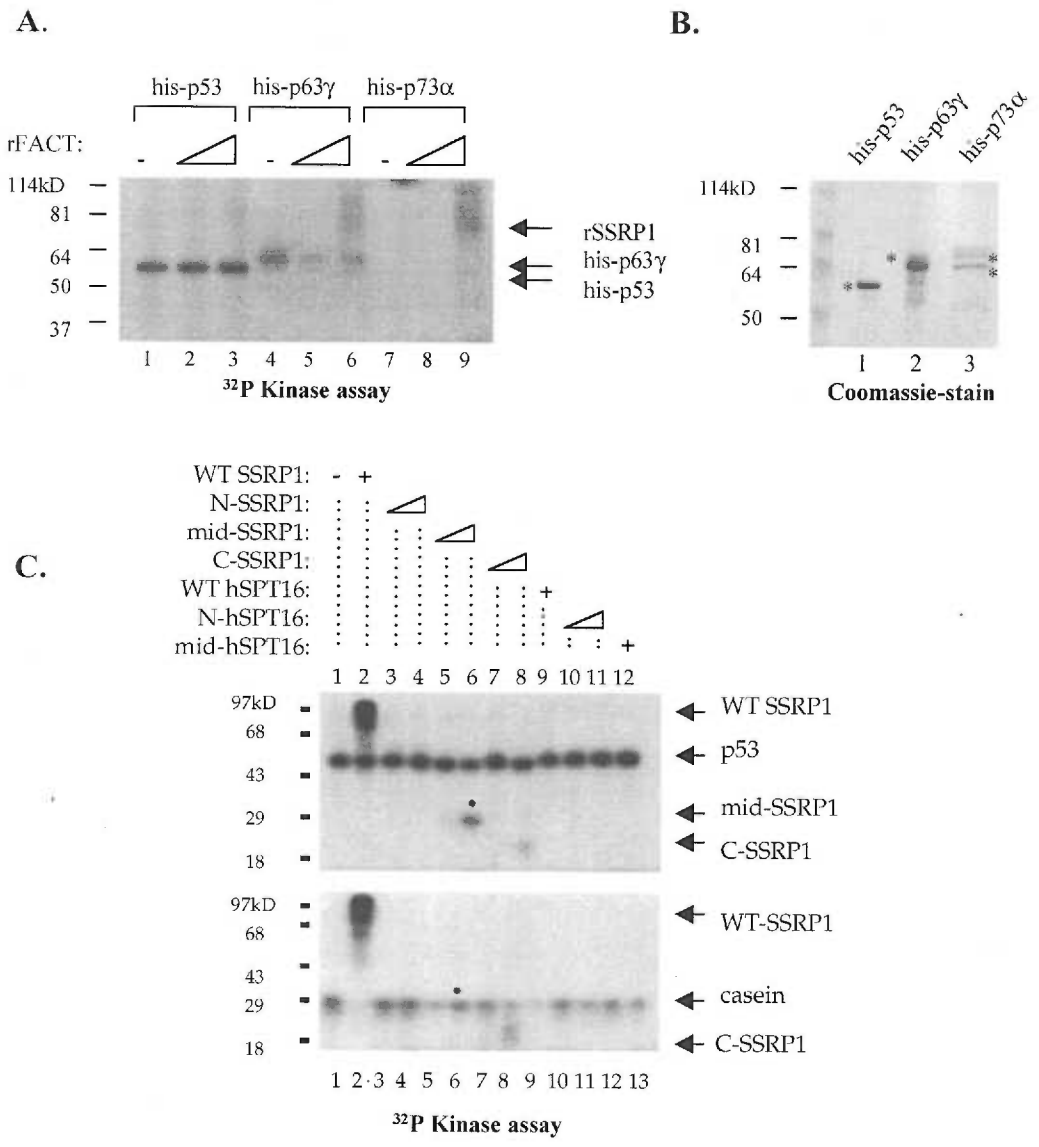
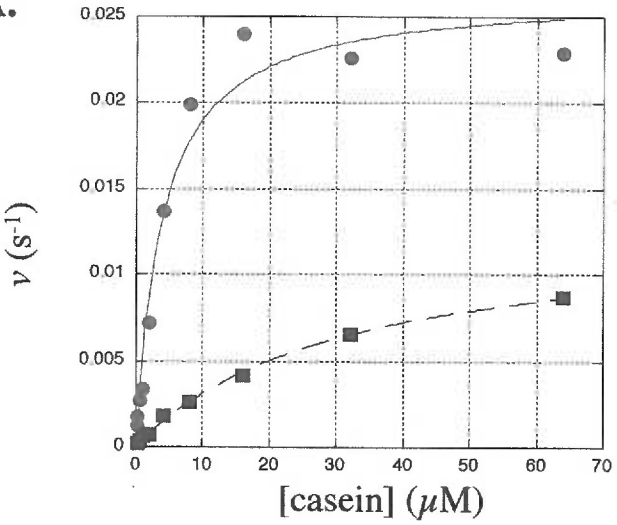
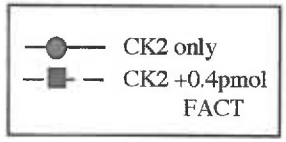

A.
his-p53
his-p63γ
his-p73α
rFACT:
114kD
81
64
50
37
1 2 3 4 5 6 7 8 9
rSSRP1
his-p63γ
his-p53
32P Kinase assay
B.
his-p53
his-p63γ
his-p73α
114kD
81
64
50
1 2 3
Coomassie-stain
C.
WT SSRP1:
N-SSRP1:
mid-SSRP1:
C-SSRP1:
WT hSPT16:
N-hSPT16:
mid-hSPT16:
1 2 3 4 5 6 7 8 9 10 11 12
97kD
68
43
29
18
WT SSRP1
p53
mid-SSRP1
C-SSRP1
97kD
68
43
29
18
WT-SSRP1
casein
C-SSRP1
1 2 3 4 5 6 7 8 9 10 11 12 13
32P Kinase assay

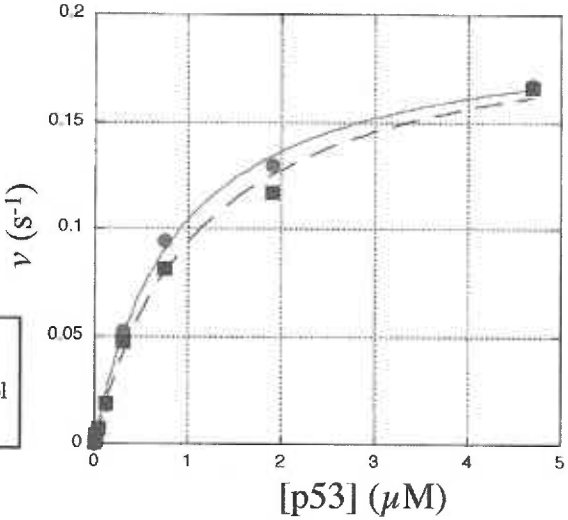
Figure 3.5. *Kinetic analysis of CK2's substrate specificity in the presence and absence of FACT.* **(A)** FACT inhibits the phosphorylation of casein by CK2. Half-hour radioactive *in vitro* kinase reactions were carried out using CK2 (0.5 units) with or without 4pmol FACT (2 pmol flag-hSPT16 + 2 pmol his-SSRP1). The kinase reactions were run by SDS-PAGE (bottom panel), the phosphorylated casein bands cut from the gel, and 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.

A.





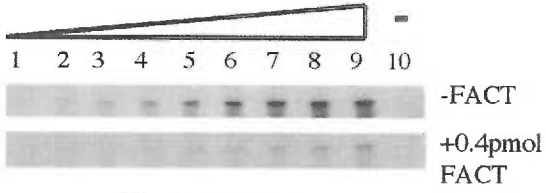
B.



	V_{max} (s^{-1})	K_m (μM)	V/K ($M^{-1}\,s^{-1}$)
-FACT	.026	3.85	6840
+FACT	.013	30.7	423

^{32}P Kinase Assay

Casein:

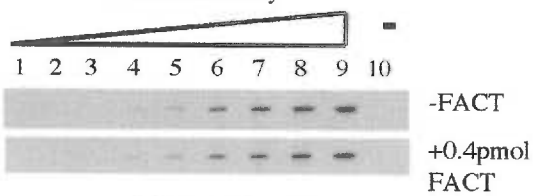


^{32}P Kinase assay

	V_{max} (s^{-1})	K_m (μM)	V/K ($M^{-1}\,s^{-1}$)
-FACT	0.20	0.89	2.2×10^5
+FACT	0.20	1.1	1.7×10^5

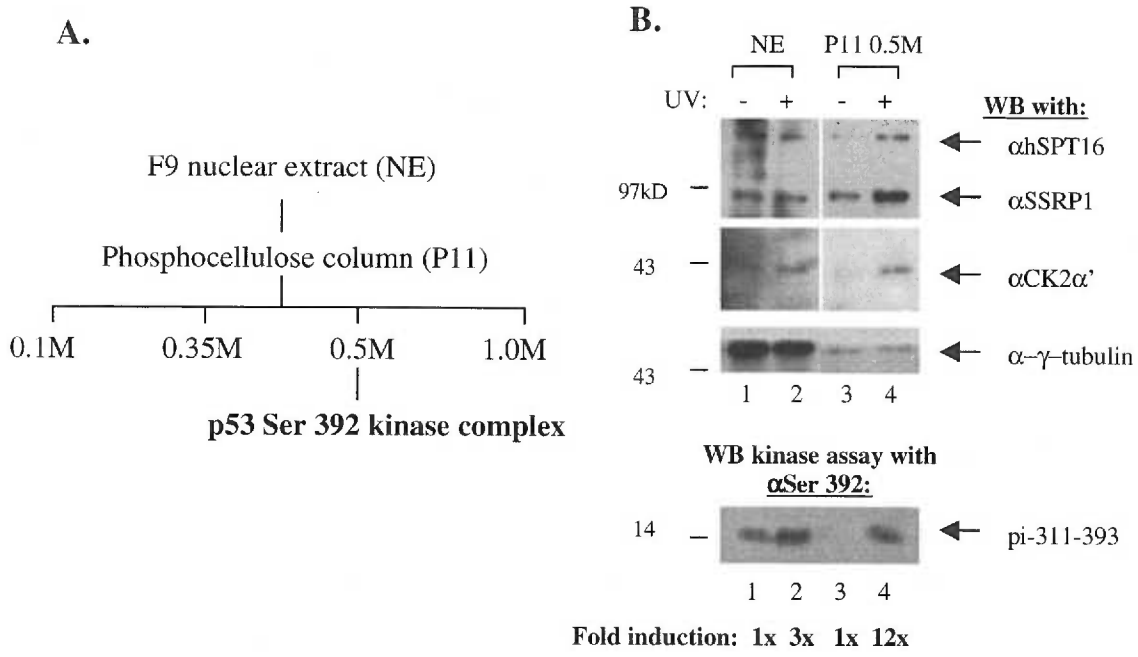
^{32}P Kinase Assay

his-p53:



^{32}P Kinase assay

Figure 3.6. *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 0.5M KCl fraction. **(B)** The p53 Ser 392 kinase complex components are enriched in the P11 0.5M KCl fraction after UV. WB analysis of nuclear extract and P11 0.5M KCl fraction from F9 cells treated with or without UV irradiation ($20J/m^2$), showing that hSPT16, SSRP1, CK2α', but not γ-tubulin are increased in the 0.5M 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 0.5M fraction. WB-kinase assay using the αSer 392 antibody showing that the Ser 392 kinase increases specific activity in nuclear extracts approximately three-fold after UV as measured by densitometry (see Experimental Procedures for details). **(C)** hSPT16, SSRP1, and CK2 associate in the UV-treated P11 0.5M fraction. Anti-SSRP1 was used for co-IP of the p53 Ser 392 kinase complex components from the -/+UV treated P11 0.5M fraction (30μg total protein). (Bottom panel) Anti-SSRP1 was also used for a IP-kinase assay using the p53 C-terminal 311-393 peptide (100ng) as a substrate and phosphorylated product was detected by WB with the αSer 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.


A.
F9 nuclear extract (NE)
Phosphocellulose column (P11)
0.1M
0.35M
0.5M
1.0M
p53 Ser 392 kinase complex
B.
NE
P11 0.5M
UV: - + - +
WB with:
97kD
αhSPT16
αSSRP1
43
αCK2α'
43
α–γ–tubulin
1 2 3 4
WB kinase assay with αSer 392:
14
pi-311-393
1 2 3 4
Fold induction: 1x 3x 1x 12x
C.
IP with: (P11 0.5M)
αSSRP1
αrabbit IgG
UV: - + +
WB with:
200kD
αhSPT16
97
αSSRP1
68
1 2 3
IP-WB kinase assay with αSer 392:
14
pi-311-393
1 2 3
D.
UV
Transcriptional stall or Replication pause
?
CK2
FACT
CK2-FACT
phosphorylation of p53 Ser 392
other CK2 substrates
Gene transcription

CHAPTER FOUR

The p38MAPK Inhibitor SB203580 Alleviates Ultraviolet-induced p53 Phosphorylation at Serine 392

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Department of Biochemistry and Molecular Biology, [2] Department of Cell and Development Biology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201; [1]University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Blvd,. Houston, TX 77030; [3]Biology Division, National Cancer Center Research Institute, Tsukiji 5-1-1, Chou-ku Tokyo, Japan.

Running title: Phosphorylation of p53 at Serine 392 by p38MAPK.

Key words: p53, p38MAPK, Serine 392 phosphorylation and UV irradiation.

*(Note: The work in this chapter was included in Keller, et al. (1999). Biochem. Biophys. Res. Comm. **261**: 464-471.)*

SUMMARY

Phosphorylation of p53 at serine 392 has been shown to be responsive uniquely to UV but not gamma irradiation, and this report describes the involvement of the UV-responsive p38MAPK protein in the phosphorylation of Ser 392. The immunoprecipitated p38MAPK from UV-irradiated murine embryonic testicular carcinoma F9 cells phosphorylated the Ser 392 residue of the human p53 protein *in vitro* and this phosphorylation was inhibited by a p38MAPK-specific chemical inhibitor SB203580. The inhibitor also remarkably alleviated the UV-caused induction of the murine p53 protein in cells. Moreover, p53 bound to p38MAPK as revealed by immunoprecipitation with anti-p53 antibodies from UV-treated F9 cells. Thus, these results suggest that UV-stimulated p53 phosphorylation at Ser 392 is mediated by the stress responsive p38MAPK.

INTRODUDCTION

UV but not γ irradiation is responsible for phosphorylation of serine 392 of the human p53 protein (corresponding to Ser 389 of murine p53) (80, 105), and enhances the sequence-specific DNA binding activity of p53 *in vitro* (70) and is important for transcriptional activation in cells (56). However, the kinase(s) responsible for this phosphorylation has not yet been identified. One of the major UV responsive pathways is the MKKK-MAP kinase cascade (reviewed in ref. 29), in which JNK1 and p38MAPK are UV-activated. Similar to JNK1 (66), the activated p38MAPK phosphorylates nuclear transcriptional activators (53, 136, 151, 162), with different substrate preferences (28). Also, p38MAPK promotes apoptosis and/or cell growth arrest (93, 168).

These characteristics of p38MAPK are similar to the UV-induced phenotype of p53 activation, leading us to study the possible relationship between the two proteins. Using immunoprecipitation followed by kinase assays with anti-p38MAPK antibodies, p38MAPK from UV-irradiated F9 or Tera-2 cells was found to phosphorylate Ser 392 of the human wild type p53 protein *in vitro*. This phosphorylation was reduced by a pyridinyl imidazole compound SB203580, which is a p38MAPK-specific inhibitor (94). In cells, this drug impaired phosphorylation at Ser 392 and induction of p53 protein levels. Finally, p38MAPK bound to p53 in cells as demonstrated by immunoprecipitation of UV-treated cells. Thus, these results suggest that p38MAPK may be one of the UV-stimulated kinases targeting the Ser 392 residue of p53.

RESULTS

p53 induction and phosphorylation at Ser 392 were repressed by a p38MAPK-specific inhibitor SB203580.

Because phosphorylation of p53 at Ser 392 is uniquely responsive to UV (80, 105) and p38MAPK is activated by UV (28) we tested whether p53 is a functional downstream component for this UV-responsive MAP kinase. A group of pyridinyl imidazole compounds was shown to specifically inhibit p38MAPK-mediated phosphorylation of several substrates with various IC50 values (28, 89, 94, 136). This inhibition further impairs the MKKK-p38MAPK signaling in response to UV irradiation (4). Thus, one p38MAPK-specific inhibitor SB203580 was employed in our study. Murine testicular carcinoma F9 cells were chosen for this test because p53 response to UV irradiation has been characterized in these cells (107). Cells were treated with 1 and 10 μM of SB203580 for two hours prior to irradiation with UV

($20J/m^2$). 8 h postirradiation, cells were harvested for Western blot analysis with anti-p53 antibody PAb421 and αS392. Consistent with our previous reports (80, 105), p53 level was induced remarkably after UV irradiation (middle panel of Figure 4.1A) and so was Ser 392 phosphorylation (top panel). Markedly, SB203580 reduced induction of either p53 level or phosphorylation at Ser 392 in a dose-dependent manner (lanes 4-6 of two top panels). This reduction was specific to p53, as equal amounts of the p38MAPK protein were detected in all three lanes (bottom panel). The p53 level was quantified in a graph in Figure 4.1B, which clearly indicated that the p38MAPK-specific inhibitor at the concentration of 10 μM can retard UV-stimulated p53 induction, suggesting that p38MAPK may mediate p53 phosphorylation at Ser 392 as well as p53 stabilization.

p38MAPK phosphorylates Ser 392 of p53.

Because SB203580 is a p38MAPK-specific inhibitor (94), the above observations suggest that p38MAPK may be the kinase that phosphorylates p53 in response to UV. To test this possibility, the p38MAPK activity was immunoprecipitated by using anti-p38 antibodies from F9 or Tera-2 cells irradiated with or without UV. Kinase activity was measured with different substrates as shown in Figure 4.2. As expected, GSTElk1, a known substrate for MAP kinases (73), was phosphorylated by UV-activated p38MAPK from F9 cells and was specifically inhibited by SB203580 (Figure 4.2A). Under the same condition, p53 was also phosphorylated by UV-activated p38MAPK (lane 6 of Figure 4.2B) and this phosphorylation was inhibited by SB203580. The phosphorylation was not catalyzed by JNK1, as this p38MAPK preparation did not possess kinase activity on GSTcJun (lane 7 of panel B). Also, the phosphorylation of p53 by p38MAPK appeared to occur at the C-terminal domain only,

because this kinase was unable to phosphorylate an alternatively spliced form of murine p53 (lane 9 of panel B), which replaces amino acid residues 364-390 with 17 different amino acids (7, 54). Thus, the UV-activated p38MAPK can phosphorylate the C-terminal domain of p53.

To further assess whether p38MAPK actually targets the p53 C-terminal domain or more specifically Ser 392, phosphorylation of the C-terminal 311-393 fragment was detected by using [γ-^{32}P]-ATP labeling and Western blot with αS392 specific to the Ser 392 phosphorylated p53 peptide (80, 105). As shown in Figure 4.3, p38MAPK indeed phosphorylated the p53 C-terminal domain (lane 5 of panel A) and particularly Ser 392 (lane 2 of panel B). Consistent with the result in Figure 4.2, this phosphorylation was also specifically inhibited by SB203580 in a dose-dependent fashion (Figure 4.3B, lanes 3-5). As a control, a newly identified Ser 392 kinase from F9 and HeLa cells in our laboratory (84) was tested for the specificity of this inhibitor. This compound was without effect on this kinase (lanes 2-3 of panel A), indicating that the newly identified kinase is not p38MAPK and SB203580 is specific to p38MAPK. Taken together, these results, consistent with the above *in vivo* observations, demonstrate that p38MAPK can directly phosphorylate the Ser 392, mediating p53 activation by UV.

p53 associates with p38MAPK in UV-irradiated F9 cells. Identification of p38MAPK as one of the p53 kinases suggests that these two proteins may associate with each other. To test this idea, a co-immunoprecipitation with anti-p53 antibodies followed by Western blot with anti-p53 or anti-p38 antibodies was conducted using UV-irradiated F9 cells. As shown in Figure 4.4, p38MAPK was co-immunoprecipitated by a monoclonal anti-p53 antibody PAb248 (lane 6), which recognizes the N-terminal

aa 14-69 of p53 (58), but not by PAb421 (lane 5), which recognizes the C-terminal aa 370-380 (58), from only the UV-irradiated F9 cells (compare lanes 3 with 6). As a negative control, an SV40 Tag-specific antibody PAb419 (58) was unable to bring down any proteins in either cases (lanes 1 and 4). This result indicates that p38MAPK may associate with the C-terminal domain but not N-terminal domain of p53, as PAb421 competed with p38MAPK for the C-terminus (lane 5), consistent with the above observations that p38MAPK phosphorylates the C-terminal Ser 392 of p53 (Figures. 4.2 and 4.3).

DISCUSSION

This report documents identification of p38MAPK as a potential p53 upstream player that phosphorylates Ser 392 in a cellular response to UV-caused DNA damage. Supporting this are several lines of evidence: (1) A p38MAPK-specific inhibitor SB203580 inhibited UV-responsive phosphorylation at Ser 392; (2) this compound delayed p53 induction and subsequently inhibited p53 transcription activity and partially impeded apoptosis (data not shown); (3) p38MAPK phosphorylated p53 at Ser 392 *in vitro* and this phosphorylation was specifically inhibited by SB203580; and (4) p38MAPK associated with p53 as shown by immunoprecipitation with anti-p53 antibodies. Thus, the UV responsive MAP kinase probably mediates p53 induction by phosphorylating Ser 392.

It is interesting that another MAP kinase participates in the UV-p53 signaling, in addition to JNK1 (44, 115). Unlike JNK1, which phosphorylates Ser 34 of p53 (115), p38MAPK specifically targets the C-terminal Ser 392 of this tumor suppressor, indicating that the UV-p53 signaling is through multiple mechanisms and more

complicated than γ-p53 signaling which can be blocked by simply deleting the ATM gene (82). The role of JNK1 appears to be more than just phosphorylation, as it was recently reported to bind to and degrade p53 in an MDM2-independent fashion when this kinase is in an inactive (dephosphorylated) form (43). Upon activation by MEKK1 or UV irradiation, JNK1 inversely stabilizes and activates p53 probably by phosphorylating it (44). It would be reasonable to see whether p38MAPK plays a similar role in regulating the stability and activity of this transcriptional activator.

Is p38MAPK the only kinase responsible for phosphorylating Ser 392 of the p53 protein in cellular response to UV irradiation? The answer would be no. First, in this study, we found that SB203580 even at higher concentration (20 μM) could not completely abolish Ser 392 phosphorylation, indicating the existence of other potential UV-responsive kinases targeting this site. In accordance with this possibility, our lab has recently identified and purified another Ser 392 kinase complex devoid of p38MAPK from F9 or HeLa cells, as analyzed by Western blot (84). Also, phosphorylation of p53 by this newly identified kinase was not inhibited by the p38MAPK-specific inhibitor (Figure 4.3A). Hence, there is more than one kinase targeting Ser 392 in response to UV. p38MAPK may present an early response by phosphorylating p53 and additionally it is possible that p38MAPK may activate other kinases that in turn directly phosphorylate p53. There are at least four isoforms of p38MAPK identified thus far (29), among which p38αn and p38β2 but not p38γ and p38δ are sensitive to SB203580 (25, 47). Thus, p38αn and/or p38β2 may phosphorylate p53. This phosphorylation may be specific to Ser 392, because this kinase did not phosphorylate the alternate spliced form of p53 (Figure 4.2B), excluding Ser 315 and the N-terminal serines, and phosphorylation at Sers 371, 376, and 378 of p53 were not

responsive to UV (164); our unpublished data).

Although phosphorylation at multiple serines of p53 occurs after UV irradiation, how cells coordinate different kinases in regulating the stability and activity of p53 needs to be further studied. One mechanism would be that this posttranslational modification at different sites provides p53 with different properties or conformations such that MDM2, a cellular p53 inhibitor (117), could no longer recognize this target. For instance, phosphorylation at Ser 15 was shown to prevent binding of MDM2 to p53 (145). MDM2-mediated degradation of p53 (61, 90) requires both the N- and C-termini of both the proteins (61, 90, 91), though their N-termini directly interact with each other (20, 21). This implicates that other unidentified cellular proteins in addition to MDM2 may be involved in regulating p53 stability probably by targeting the C-terminus of p53. Therefore, phosphorylation at the C-terminal residues may effect targeting of p53 by those molecules or ubiquitination of p53 (67). Although this notion remains to be tested, it is clear that diverse pathways mediate stress responseive p53 activation (46). This phenomenon is correlated well with the central importance of this tumor suppressor in monitoring cell growth so that it is still functional when one mechanism, such as phosphorlyation (3, 9), of the activating pathways becomes defective. Thus, molecular dissection of the sophisticated UV-p53 signaling would provide much insight into understanding of cellular networking in finely tuning p53 function.

MATERIALS AND METHODS

Reagents and Buffers. The pyridinyl imidazole compound SB203580 was purchased from CalBiochem, Inc (CA). Western blotting lysis buffer is composed of 50 mM Tris/HCl (pH 8.0), 0.5% NP-40, 5 mM EDTA, 2 mM DTT, 150 mM NaCl and 0.2

mM PMSF. SNNTE is composed of 50 mM Tris/Hcl (pH 7.4), 5 mM EDTA, 1% NP-40, 500 mM NaCl and 5% sucrose. RIPA is comprised of 50 mM Tris/Hcl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, and 1% (w/v) sodium deoxycholate. Immunoprecipitation-kinase lysis buffer is 100mM Hepes-KOH (pH 7.4), 2mM EGTA, 50mM β-glycerophosphate, 10% glycerol, and 1% Triton X-100. LiCl buffer is 500mM LiCl, 100mM Tris/HCL (pH 7.6), and 0.1% Triton X-100. p38MAPK assay buffer is 10mM Mops (pH 7.2), 25mM $MgCl_2$, 2mM EGTA, 0.1% Triton X-100, and 1μCi [γ-^{32}P]-ATP.

Plasmids and antibodies. pGST-Elk1 and pGSTcJun were as described (72). Polyclonal rabbit anti-p38 and anti-JNK1 antibodies were purchased from Santa Cruz (CA). The polyclonal anti-p38 antibody (#12850) for immunoprecipitation was provided by Gary Johnson (73). Antibodies αS392 specifically against the phosphorylated Ser 392-containing p53 C-terminal peptides were generated as previously described (80, 105, 145). The anti-p53 antibody PAb421, PAb248, and anti-Tag antibody PAb419 were as described (176).

Cell lines and cell culture. Human teratoma tera-2 cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 0.1 mg/ml streptomycin at 37^{O}C in a 5% CO_2 atmosphere. DMEM without sodium pyruvate was used for murine testicular carcinoma F9 cells. F9 and Tera-2 cells contain wild type p53.

Preparation of F9 or Tera-2 nuclear extracts and purification of human p53 and mouse alternate splice p53 proteins. F9 or Tera-2 cell nuclear extracts were prepared and purification of human p53 and mouse alternate splice p53 proteins were purified as previously described (103).

UV irradiation and chemical treatment. UV irradiation (20 J/m^2) of cells was carried out using the 254 nm source of a UV transilluminator (UVP, Inc, Upland, CA), as described (73, 105). Cells were incubated with different concentrations of the p38MAPK inhibitor SB203580 as indicated in figure legends for two h prior to UV irradiation. Postirradiation, cells were harvested either at different time points as indicated in figure legends or 8 h afterward for immunoprecipitation-Western blot, or kinase assays.

Immunoprecipitation-kinase assay. Immunoprecipitation-kinase assays were performed using described methods (73, 103). Briefly, cell lysates containing approximately 500 μg of proteins were incubated with 3 μl of polyclonal rabbit anti-p38 antibodies at 4°C for 1 h, followed by incubating with 30 μl of protein A-agarose (50 % slurry, Pharmacia) for 1 h. Agarose beads were then washed with lysis buffer once, LiCl buffer once, and kinase assay buffer once. Reaction mixtures were in 20μl assay buffer including the substrates in the presence or absence of SB203580 (see figure legends for amounts of the drug and substrates used). The reaction mixtures were incubated at 30°C for 30 min and stopped by addition of protein sample loading buffer. Phosphorylated protein signals were detected by autoradiography after analysis on an SDS-gel.

Immunoprecipitation-Western blot. Western blot analysis was performed as described (105). Proteins from either immunoprecipitation (500 μg) or straight cell lysates (100 μg) of the UV-treated or untreated cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The membrane was immunoprobed with PAb421, and αS392 as shown in Figure 4.1, or with polyclonal anti-p38 or anti-p53 antibodies for Figure 4.4. Signals were detected by the ECL reagents (Santa Cruz Inc, CA). The relative quantification of p53 levels was

conducted using Model GS-700 Imaging Densitometer (BioRad).

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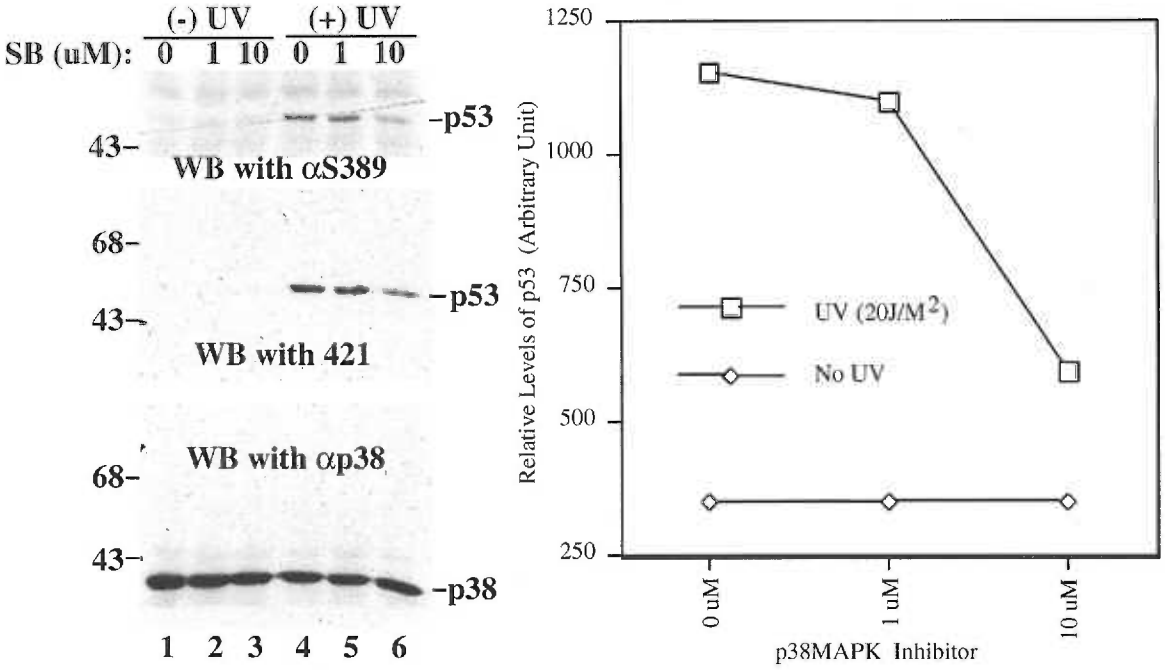

(-) UV
(+) UV
SB (uM): 0 1 10 0 1 10
-p53
43-
WB with αS389
68-
-p53
43-
WB with 421
WB with αp38
68-
43-
-p38
1 2 3 4 5 6
1250
1000
750
500
250
Relative Levels of p53 (Arbitrary Unit)
UV (20J/M2)
No UV
0 uM
1 uM
10 uM
p38MAPK Inhibitor

Figure 4.2. *p38MAPK phosphorylates GSTELK1 and wt p53 in vitro and is inhibited by SB203580.* **(A)** UV-activated p38MAPK phosphorylates Elk1. F9 cell lysates ($5x10^6$ cells) treated with or without UV ($20J/m^2$) were prepared and incubated with anti-p38 antibodies (αp38) and protein A-agarose, as described in Materials and Methods. 20 μl kinase reactions were carried out with 2.5 μg of GSTElk1 (GElk1) and [γ-^{32}P]-ATP as substrates. Lysate sources for kinases with or without UV-irradiation as well as SB203580 (SB) were indicated on top. Signals were detected by autoradiography. **(B)** p38MAPK does not phosphorylate an alternate splice form of murine p53 (p53AS) or GSTcJun (GJun). 100 ng of WT p53 and 1 μg of GELK1, 1.0 μg GcJun or 100ng p53AS were used as indicated on top.

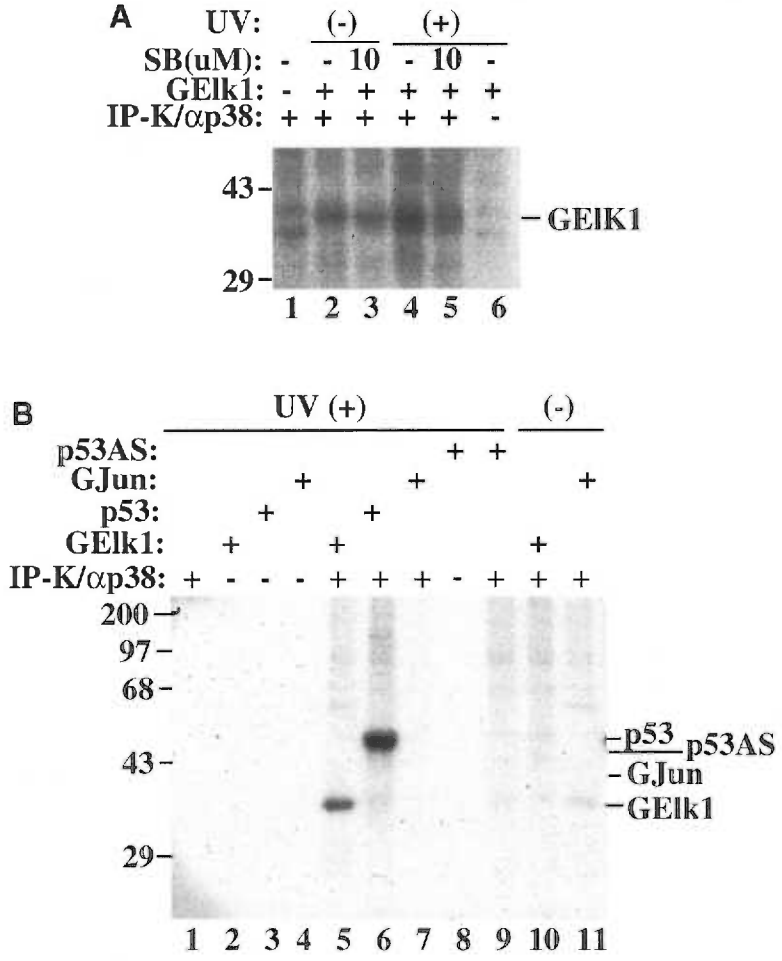

A
UV: (-) (+)
SB(uM): - - 10 - 10 -
GElk1: - + + + + +
IP-K/αp38: + + + + + -
43
29
GElK1
1 2 3 4 5 6
B
UV (+) (-)
p53AS: + +
GJun: + + +
p53: + +
GElk1: + + +
IP-K/αp38: + - - - + + + - + + +
200
97
68
43
29
p53
p53AS
GJun
GElk1
1 2 3 4 5 6 7 8 9 10 11

Figure 4.3. *p38MAPK phosphorylates the C-terminal Ser 392 of the human p53.* **(A)** Phosphorylation of the p53 C-terminal domain by p38MAPK is inhibited by SB203580. A similar kinase assay to the above ones was carried out, but the p53 C-terminal 311-393 fragment (100 ng) was used as a substrate. Besides p38MAPK, another newly identified kinase temporarily named 392K (100 ng) was also included as a control. The amount of SB203580 used is indicated on top. **(B)** p38MAPK phosphorylates Ser 392, which is also sensitive to SB203580. The same kinase assay as that in panel A was performed except non-labeled ATP was used. The phosphorylated human p53 C-terminal peptides were detected by Western blot with αS392. The amounts of SB203580 are indicated on top.

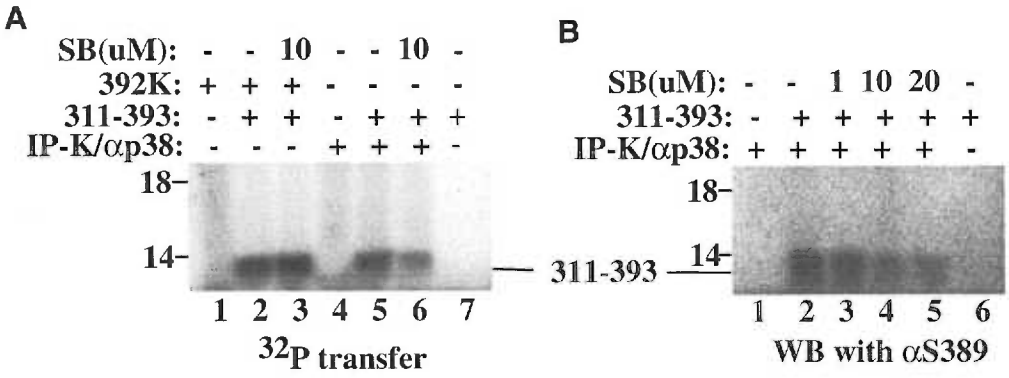
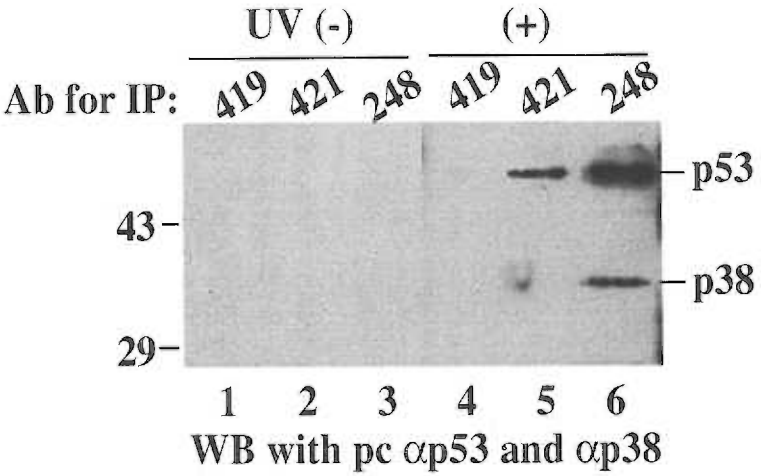

A
SB(uM): - - 10 - - 10 -
392K: + + + - - - -
311-393: - + + - + + +
IP-K/αp38: - - - + + + -
18-
14-
1 2 3 4 5 6 7
32P transfer
B
SB(uM): - - 1 10 20 -
311-393: - + + + + +
IP-K/αp38: + + + + + -
18-
311-393
14-
1 2 3 4 5 6
WB with αS389

Figure 4.4. *p38MAPK interacts with p53 in vivo following UV irradiation.* Lysates (500 μg proteins) from F9 cells either exposed to UV light (10J/m^2) or not were incubated with 2μg of PAb419, PAb421 or PAb248 antibodies for immunoprecipitation, as described in Materials and Methods. After transferred to nitrocellulose membrane, p53 or p38 was detected by Western blot with polyclonal anti-p53 or anti-p38 antibodies, as indicated at the bottom.


UV (-)
(+)
Ab for IP:
419
421
248
419
421
248
p53
p38
43
29
1
2
3
4
5
6
WB with pc αp53 and αp38

CHAPTER FIVE

SUMMARY AND CONCLUSIONS

Because phosphorylation of the p53 tumor suppressor protein at serine 392 is highly conserved and is important for its function, and because it is phosphorylated in cells after treatment with genotoxic agents such as UV irradiation and cisplatin, this thesis dissertation seeks to identify the p53 Ser 392 kinase and in the process perhaps uncover a novel stress-induced p53-activating pathway. Here it is shown that the UV-responsive kinase is CK2, a previously identified p53 Ser 392 kinase, providing further validation of a role for this kinase in p53 regulation. Surprisingly, CK2 is regulated as part of a novel protein complex with the chromatin-associated factors, hSPT16 and SSRP1. Together hSPT16 and SSRP1 form a heterodimer (known in mammals as FACT) that when bound to CK2 influences its substrate specificity such that it phosphorylates p53 Ser 392 over other tested substrates, including even the p53 family member, p63γ. Also, it is shown that phosphorylation of p53 Ser 392 enhances its sequence-specific DNA binding and transactivation functions *in vitro* and in cells.

Because the hSPT16-SSRP1 heterodimer is highly conserved in eukaryotes and little is known about its basic biochemistry, and because of its novel interaction with CK2, this dissertation investigates their protein-protein interactions. CK2, hSPT16, and SSRP1 bind to each other via non-overlapping domains, consistent with the idea that they form a protein complex. Also, hSPT16 and SSRP1 inhibit CK2's ability to phosphorylate non-p53 substrates in a manner that may reflect an induced conformational change in the protein.

Here it is shown that the specific activity of the p53 Ser 392 kinase increases following UV, and evidence is presented that the CK2-hSPT16-SSRP1 complex

increases as well. The data supports two models for how this complex is regulated after DNA damage thus leading to phosphorylated p53 Ser 392 (see Figure 3.6D), though these models may co-exist. First, CK2's specific activity increase following UV irradiation may be due to activation by the stress-responsive p38MAPK. It is shown that a p38MAPK inhibitor represses p53 induction and Ser 392 phosphorylation after UV treatment. Furthermore, p38MAPK has been shown to stimulate CK2's ability to autophosphorylate, perhaps reflecting an increase in its specific activity (142). A second model for the CK2-hSPT16-SSRP1 complex activation is that it may bind to damaged DNA directly and become activated, by virtue of SSRP1's ability to bind to cisplatin-modified DNA (14).

In conclusion, the results presented in this dissertation uncover a new p53-activating pathway, and just as importantly they substantiate and expand upon previous inconclusive evidence regarding the role of CK2 phosphorylation of p53 Ser 392. Though CK2 was the first p53 kinase identified, its role in p53 biology has always been controverial because in most systems it is constitutively active and not stress-responsive. The evidence presented here resolves this mystery, by identifying regulatory proteins that associate with CK2 and modify its kinase activity after UV-induced DNA damage.

Furthermore, discovering a new cellular protein complex opens up new research areas into its functions beyond p53. This is especially intriguing because the CK2-SPT16-SSRP1 complex has been identifed in *S. cerevisiae* (45) which does not contain p53, and thus would be expected to play regulatory roles in undetermined ways. It is possible to imagine that CK2 would affect the function of hSPT16 and SSRP1, perhaps in its role in transcription and replication elongation through chromatin, or in its role in

DNA damage recognition. One of the most important areas of research in a post-genome era is that of identifying how the products of these genes interact with each other in the cell to form highly connected cellular networks. In this regard, this dissertation makes a small, but significant, contribution.

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