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p53 Is Phosphorylated by CDK7-Cyclin H in a p36^{MAT1}-Dependent Manner

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The tumor suppressor protein p53 acts as a transcriptional activator that can mediate cellular responses to DNA damage by inducing apoptosis and cell cycle arrest. p53 is a nuclear phosphoprotein, and phosphorylation has been proposed to be a means by which the activity of p53 is regulated. The cyclin-dependent kinase (CDK)-activating kinase (CAK) was originally identified as a cellular kinase required for the activation of a CDK-cyclin complex, and CAK is comprised of three subunits: CDK7, cyclin H, and p36^{MAT1}. CAK is part of the transcription factor IIH multiprotein complex, which is required for RNA polymerase II transcription and nucleotide excision repair. Because of the similarities between p53 and CAK in their involvement in the cell cycle, transcription, and repair, we investigated whether p53 could act as a substrate for phosphorylation by CAK. While CDK7-cyclin H is sufficient for phosphorylation of CDK2, we show that p36^{MAT1} is required for efficient phosphorylation of p53 by CDK7-cyclin H, suggesting that p36^{MAT1} can act as a substrate specificity-determining factor for CDK7-cyclin H. We have mapped a major site of phosphorylation by CAK to Ser-33 of p53 and have demonstrated as well that p53 is phosphorylated at this site in vivo. Both wild-type and tumor-derived mutant p53 proteins are efficiently phosphorylated by CAK. Furthermore, we show that p36 and p53 can interact both in vitro and in vivo. These studies reveal a potential mechanism for coupling the regulation of p53 with DNA repair and the basal transcriptional machinery.

The tumor suppressor protein p53 is a transcriptional activator that mediates cellular responses to genotoxic stress (20, 30, 35). The accumulation of p53 after DNA damage can cause cell cycle arrest in G_1 by its transcriptional activation of the *p21* gene (12), which encodes a potent inhibitor of cyclin-dependent kinases (CDKs). By causing cells to arrest in G_1, p53 may allow time for damaged DNA to be repaired, thus avoiding the propagation of potentially deleterious mutations. Alternatively, p53 mediates apoptosis in response to DNA damage in some cells, in another mechanism by which it serves to ensure genomic integrity.

A direct role for p53 in DNA repair has been suggested by its association with mismatched and damaged DNA (32, 49) as well as with certain proteins required for repair, such as the single-stranded DNA binding protein RP-A (11, 22, 36) and the transcription factor IIH (TFIIH) components XPB (ERCC3), XPD (ERCC2), and p62 (34, 65, 66, 69). Notably, the sequence-specific DNA binding activity of p53 is also strongly stimulated by the redox-repair protein Ref-1 (27) as well as by short single-stranded DNA of the size that is generated during nucleotide excision repair (28). However, there have been conflicting reports as to the repair phenotype of p53-deficient cells. While a deficiency of p53 led to reduced repair of DNA in some cases (18, 21, 58, 66), Li-Fraumeni cells, which have only a single wild-type allele of p53, showed normal transcription-coupled repair (18) and p53 −/− mouse fibroblasts showed normal rates of repair compared to wild-type cells (25, 52).

The p53 protein can be divided into approximately three portions: (i) the N-terminal domain, containing the activation domain; (ii) the central, sequence-specific DNA binding domain; and (iii) the multifunctional, regulatory C-terminal domain (for a review, see references 20 and 30). The activation domain interacts with components of the basal transcriptional machinery, including TBP, $TAF_{II}31$, and $TAF_{II}80$ (38, 63; see reference 13 and references therein) as well as the p62 polypeptide component of TFIIH. Residues 22 and 23 are critical for the interactions of p53 with the TAFs (38, 63). The MDM2 protein, which acts as a repressor of p53 transcriptional activation, also associates with this region of p53, and substitution of residues 22 and 23 disrupts this interaction (31, 37).

p53 is phosphorylated in its N- and C-terminal domains by a variety of kinases. Casein kinase I, double-stranded DNA-dependent protein kinase (DNA-PK), Jun N-terminal kinase (JNK), and MAP kinases phosphorylate p53 in its N terminus, and CDKs, protein kinase C (PKC), and casein kinase II phosphorylate p53 in its C terminus (42; see reference 40 for a review). Phosphorylation of its C terminus by CDKs, PKC, or casein kinase II stimulates the sequence-specific DNA binding ability of p53 in vitro (23, 24, 60, 67). Although hyperphosphorylation of p53 correlates with its increased transcriptional activating ability (72), in vivo analysis of mutant versions of p53 with substitutions at phosphate acceptor groups has resulted in conflicting conclusions as to the role of these phosphorylation sites (see references 20 and 30).

The sequential activity of CDKs regulates cellular transit through the cell cycle (44, 53). The CDKs, in turn, are regulated both positively and negatively by interactions with various proteins as well as by their phosphorylation state (44). Optimal activation of a CDK requires (i) binding by its appropriate cyclin partner; (ii) the absence of CDK inhibitors, such as the

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p16^{INK4A}-p15^{INK4B} family or the p21-p27^{Kip1} family; (iii) removal of inhibitory phosphate groups at residues corresponding to Thr-14 and Tyr-15 of CDC2; and (iv) phosphorylation in the activating T loop, corresponding to Thr-161 in CDC2. The enzymatic activity responsible for this stimulatory phosphorylation is the CDK-activating kinase (CAK).

CAK was purified from *Xenopus* oocytes, starfish oocytes, and mammalian cells (15, 46, 59) and found to contain a catalytic subunit that had previously been cloned as MO15 (57). Because of its structural similarity to other CDKs, MO15 was renamed CDK7. Like other CDKs, CDK7 has its own cyclin partner, cyclin H, which is also a component of CAK. In contrast to other CDKs, CAK also contains a third polypeptide subunit, p36^{MAT1}, which was identified by its copurification with CDK7 and cyclin H. The amino acid sequence derived from the cloned p36 cDNA predicts a C_3HC_4 RING finger in the N terminus of the protein, but it otherwise shows no homology to known sequences (9, 17, 61, 71). p36 acts as an assembly factor for CDK7 and cyclin H (9, 17, 61).

In cells, CAK exists either free or in association with the multiprotein complex TFIIH (1). The identification of CDK7 as a component of TFIIH (14, 51, 54, 56) shed new light on the potential roles of this kinase, since TFIIH is required for basal transcription by RNA polymerase II as well as nucleotide excision repair (10, 68; for a review, see reference 55). TFIIH-associated CAK can phosphorylate the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II in its YSPTSPS repeats (54), and phosphorylation of the CTD is believed to be important in the promoter clearance step of transcription. It is notable that CDK7 is able to phosphorylate both the T loop of CDKs and the CTD repeats, since there is no obvious sequence similarity near their respective phosphate acceptor sites. Isolation of TFIIH from HeLa cells has demonstrated the presence of two discrete complexes of TFIIH, one of which is comprised of CDK7, cyclin H, p36, and ERCC2 (48). How the presence of ERCC2 might affect CAK functioning is presently not known.

Recent reports have yielded new insight into the regulation of CDK7-cyclin H activity by association with p36^{MAT1} and TFIIH. The presence of p36^{MAT1} serves as an alternate mechanism for the activation of CDK7, circumventing the need for the activating phosphorylation at the T loop of CDK7 itself at Thr-170 (17). Additionally, p36^{MAT1} has recently been shown to determine the substrate specificity of CDK7-cyclin H. CTD substrates are preferred over CDK2 substrates for CDK7-cyclin H specifically in the presence of p36^{MAT1}. At a further level of regulation, the association with TFIIH also affects the kinase activity of CAK, both qualitatively and quantitatively. In results similar to those obtained in the presence of p36^{MAT1}, TFIIH-associated CAK preferentially phosphorylates CTD substrates over CDK2 substrates, as compared to free CAK (50, 70). Finally, a differential response of TFIIH-associated CAK activity compared with free CAK activity was observed after UV irradiation of cells (1). Thus, the initially surprising result that CAK activity, which would be expected to be cell cycle regulated, remains constant throughout the cell cycle (5, 47, 62) may be explained with further investigations into the complexities of regulation of CAK activity.

Given that both p53 and CAK have been either demonstrated or strongly implicated as having roles in the common cellular processes of cell cycle regulation, transcription, and DNA repair, we were interested in investigating whether p53 could act as a substrate for phosphorylation by CAK. We have found that CDK7-cyclin H can phosphorylate p53 in a manner that is strongly stimulated by p36^{MAT1}. We have identified a major phosphorylation site of CAK within the N terminus of p53, specifically at Ser-33. Furthermore, we have shown that p36^{MAT1}, like other polypeptides of TFIIH, can physically associate with p53. CAK phosphorylation of p53 may therefore provide a mechanism by which p53 may be regulated by the basal transcriptional and/or DNA repair machinery.

MATERIALS AND METHODS

Bacterium-expressed proteins. The p53 cDNA was cloned into the pRSETB vector (Invitrogen) for overexpression in BL21-DE3 cells. Overnight cultures were diluted, induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), and grown at 37°C for 3 h. Cells were pelleted, resuspended in phosphate-buffered saline (PBS), and sonicated, followed by centrifugation at 10,000 × *g* for 20 min. The insoluble His-tagged p53 (Hisp53) protein that was found in the pellet was resolubilized in 6 M guanidine HCl and then gradually diluted with 100 mM Tris HCl (pH 8.0)–1 mM EDTA–3 mM reduced glutathione–0.3 mM oxidized glutathione to a final guanidine HCl concentration of 1.2 M. The renatured Hisp53 was then dialyzed against 20 mM Tris HCl (pH 8.0)–50 mM KCl–1 mM EDTA, against 20 mM Tris HCl (pH 8.0)–100 mM KCl–1 mM EDTA, and finally against 20 mM Tris HCl (pH 8.0)–100 mM KCl–1 mM dithiothreitol (DTT). Hisp53 was then purified over Ni-nitrilotriacetic acid (NTA)–agarose beads (Qiagen), washed with 20 mM imidazole, and eluted with 200 mM imidazole.

Deletions of p53 were made by digesting the p53 cDNA as follows: 31-393 with *Psp*1406I and *Xba*I; 42-393 with *Bsr*DI and *Xba*I; 1-157 with *Nco*I-*Nco*I; 160-306 with *Nco*I-*Cac*I; 1-375 with *Xba*I and *Acc*I; 160-393 with *Nco*I-*Xba*I; and 160-375 with *Nco*I-*Acc*I. Each portion of p53 was cloned into the pRSETB vector transformed into BL21-DE3 cells. Induced proteins were purified over Ni-NTA–agarose as described above.

GSTp53, GST143, GST175, GST273, and GST1-82 were constructed and purified as described previously (13) with glutathione-Sepharose beads (Pharmacia).

p53 core (96-312) and tetra+basic (311-393) peptides were a gift from N. Pavletich (45).

Human His-tagged p36^{MAT1} (Hisp36^{MAT1}) was overexpressed in BL21-DE3 cells. Freshly transformed bacteria were grown overnight. After dilution of cultures to 1:100, cultures were grown at 37°C for 2 h to an optical density at 600 nm of 0.5. IPTG was added, and the mixture was incubated overnight. The cells were pelleted and lysed in 40 ml of ice-cold binding buffer B (50 mM Tris HCl [pH 8.0], 0.4 M NaCl, 5 mM imidazole) plus 1 mg of lysozyme per ml for 10 min on ice. After sonication and centrifugation at 38,000 × *g* and 4°C for 20 min, the pellets, containing more than 90% of total Hisp36^{MAT1} expressed, as judged by Coomassie blue staining following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), were resuspended in 5 ml of buffer B plus 6 M guanidine HCl and incubated on ice for 60 min. After centrifugation at 38,000 × *g* and 40°C for 20 min, the resulting supernatant (~160 mg of protein) was incubated with 0.5 ml of nickel resin (Invitrogen) preequilibrated with buffer B plus 6 M guanidine HCl. The mixture was rocked at 4°C for 2 h. The Ni beads were then washed sequentially with 40 ml of buffer B plus 6, 3, 1, or 0 M guanidine HCl. Bound protein (3.8 mg) was eluted with buffer B containing 0.5 M imidazole. The eluate was then directly loaded onto a hydroxyapatite column (1.0 by 0.6 cm; 0.5 ml) preequilibrated with buffer C (10 mM KPO_4 [pH 7.0], 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.5 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.2 μg of antipain per ml, 0.1 μg of leupeptin per ml). After the column was washed with 10 ml of buffer C, the bound protein was eluted with 15 ml of a linear gradient of 10 to 500 mM KPO_4 in buffer C. The peak of p36^{MAT1} activity (~0.5 mg) eluted at about 100 mM KPO_4. Cyclin A and GST-CDK2 were prepared as described previously (8).

Baculovirus-expressed proteins. Wild-type and tumor-derived mutant p53 proteins expressed from baculovirus-infected cells were affinity purified with monoclonal antibody PAb 421 beads as described previously (2, 19), except for the CΔ30 mutant, which was purified with its hemagglutin tag and monoclonal antibody 12CA5 beads (26).

The cDNA encoding the 15A37A double mutant was generated by swapping part of the 15A and 37A cDNAs (16) (kindly provided by M. Fiscella and E. Appella, National Institutes of Health). The mutant cDNA was cloned into the pBacPAK8 vector (Clontech) and used to produce baculoviruses in Sf9 cells. 15A37A protein was immunopurified through a PAb 421 column.

For large-scale infection with CDK7- and cyclin H-producing baculoviruses and preparation of the CDK7-cyclin H complex, monolayer High Five (HF) insect cells (Invitrogen) contained in 20 flasks (150 cm^2) and maintained in Grace's medium supplemented with 10% fetal bovine serum at 70 to 80% confluency were infected simultaneously with recombinant baculoviruses that produce CDK7 or cyclin H for 48 h at 27°C. To harvest, the cells were centrifuged at 180 × *g* for 12 min followed by one wash with ice-cold PBS. The cell pellet was then resuspended in 20 ml of hypotonic buffer (10 mM Tris HCl [pH 7.4], 25 mM NaCl, 1 mM EDTA, 5 mM DTT, 0.1 mM PMSF, 1 g of antipain per ml, 1 g of leupeptin per ml) and lysed with 20 strokes of a Dounce homogenizer. After centrifugation at 38,000 × *g* and 4°C, the resulting supernatant (60 mg of protein) was directly loaded onto a Q-Sepharose column (1.0 by 1.3 cm; 1.5 ml) preequilibrated with buffer A (25 mM Tris HCl [pH 7.5], 1 mM EDTA, 0.01%

Nonidet P-40 [NP-40], 1 mM DTT, 10% glycerol, 0.1 mM PMSF, 0.2 μg of antipain per ml, 0.1 μg of leupeptin per ml) plus 0.025 M NaCl. After the column was washed with 30 ml of equilibration buffer (buffer A plus 0.025 M NaCl), the bound protein was eluted with 40 ml of a linear gradient of 0.025 to 0.4 M NaCl in buffer A. The peak of CDK7-cyclin H complex activity, monitored by silver staining following SDS-PAGE, eluted at about 0.18 M NaCl. The pooled CDK7-cyclin H fractions were then diluted twofold with buffer A prior to chromatography on a heparin-Sepharose column (1.0 by 2.5 cm; 2 ml) preequilibrated with buffer A plus 0.1 M NaCl. After the column was washed with 100 ml of equilibration buffer, the bound protein was eluted with 40 ml of a linear gradient of 0.1 to 1 M NaCl in buffer A. The CDK7-cyclin H complex eluted at about 0.35 M NaCl, with a yield of ~2 mg with more than 90% purity, as judged by silver staining following SDS-PAGE.

Kinase assays. Kinase assays were performed with approximately 100 ng of substrate and 100 ng each of CDK7, cyclin H, and Hisp36^{MAT1} in 10-μl reaction mixtures containing 50 mM Tris HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 100 μg of bovine serum albumin (BSA) per ml, 50 μM ATP, and 2.5 μCi of [γ-^{32}P]ATP. Reaction mixtures were incubated at 37°C for 30 min, and the reactions were then stopped with SDS loading dye. Reaction mixtures were run on SDS–10% polyacrylamide gels, fixed, silver stained, and then allowed to dry before autoradiography. Quantitation was performed on a Molecular Dynamics PhosphorImager.

Two-dimensional phosphotryptic mapping. Two-dimensional phosphotryptic mapping was performed as described previously (4). Hisp53 (300 ng) was phosphorylated in vitro in the presence of [γ-^{32}P]ATP, electrophoresed by SDS-PAGE, and transferred to nitrocellulose. Labeled proteins were then oxidized with performic acid and subjected to trypsin digestion. After repeated lyophilization, pellets were resuspended in 10 μl of pH 1.9 buffer (50 ml of formic acid [88%], 156 ml of glacial acetic acid, 1,794 ml of deionized water). Samples were subjected by Cerenkov counting, and 2,000 to 5,000 counts were spotted on thin-layer chromatography plates (EM Science, Gibbstown, N.J.) and run on a Hunter thin-layer peptide mapping system (CBS Scientific, Del Mar, Calif.) in the first dimension with pH 1.9 buffer for 40 min at 1 kV. The counts were run in the second dimension with isobutyric acid buffer (1,250 ml of isobutyric acid, 38 ml of *n*-butanol, 96 ml of pyridine, 58 ml of glacial acetic acid, 558 ml of deionized water) until the samples were approximately 3 cm from the top of the plates. Plates were dried and exposed to film at −70°C for 2 days to 1 week. Mobility predictions were made as described previously (4).

In vitro translation and binding assays. To prepare DNA allowing the expression of the C-terminally truncated p36^{MAT1} protein His1-253, a DNA fragment containing the sequence coding for p36^{MAT1} amino acid residues 1 to 253 was generated by PCR with pET-MAT1 (wild-type) plasmid DNA as a template. The sequence of the N-terminal PCR primers used to amplify p36^{MAT1} 1-253 was 5′ GGA GGG AGA ATT CCA TGG. The sequence of the C-terminal primer used was 5′ ATG TGG TCG ACA TGT CTC. The PCR products were digested with *Eco*RI and *Sal*I prior to ligation with pHT7MAT1 plasmid DNA (a gift from S. Inamoto and R. Roeder, Rockefeller University) which had been *Eco*RI and *Sal*I digested.

To prepare DNAs allowing the expression of the C-terminal p36^{MAT1} protein His205-309, DNA fragments containing the sequence coding for the p36^{MAT1} C-terminal 103 amino acids were generated by PCR with pET-MAT1 (wild-type) plasmid DNA as a template. The sequence of the N-terminal PCR primers used to amplify 205-309 was 5′ AGA GAA TTC TAC CCA ATT AG. The sequence of the C-terminal primer used was 5′ CTG GGT CGA CTC CAA GGT. The PCR products were digested with *Eco*RI and *Sal*I prior to ligation with pHT7MAT1 plasmid DNA which had been *Eco*RI and *Sal*I digested. Both p36^{MAT1} His1-253 and p36^{MAT1} His205-309 contain a His tag leader sequence (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRDPN) at the N terminus.

^{35}S-methionine-labeled full-length and deletion mutant p36^{MAT1} proteins were produced in rabbit reticulocyte lysates (TNT; Promega) and used in in vitro binding assays with Hisp53. Five microliters of in vitro-translated p36^{MAT1} was incubated with 1 μg of Hisp53 in 25 mM Tris HCl (pH 7.5)–1 mM EDTA–1 mM DTT–0.01% NP-40–10% glycerol–0.5 mM PMSF–0.1 mM benzamidine–300 μg of leupeptin per ml–10 μg of bacitracin per ml–100 μg of α-macroglobulin per ml for 30 min at room temperature. Twenty-five microliters of a 50% slurry of Sepharose beads cross-linked with anti-p53 antibodies (PAb 421, PAb 1801, and PAb DO-1) was then added, and the mixture was rotated for 2 h at room temperature. The beads were then washed four times in 200 mM NaCl–50 mM Tris HCl (pH 7.2)–0.1% NP-40 and resuspended in protein sample buffer. Samples were run on SDS–15% polyacrylamide gels, fixed in 25% isopropyl alcohol–1% acetic acid for 30 min, washed in double-distilled H_2O for 10 min, amplified in 1 M salicylic acid for 30 min, dried, and exposed.

Full-length and deletion constructs of p53 used for in vitro translation were described previously (26). Assays similar to those described above for p36^{MAT1} were performed with in vitro-translated p53, with the following exceptions. Ten microliters of in vitro-translated p53 was used with 700 μg of Hisp36 in 30-μl reaction mixtures under the same conditions as those described above, but without EDTA and DTT. After 30 min of incubation at room temperature, imidazole was added to a final concentration of 40 mM; 20 μl of a 50% slurry of Ni-NTA–agarose beads was added to the reaction mixture, which was allowed to incubate at 4°C for 1 h with rotation. The beads were then washed three times with RIPA buffer with 40 mg of BSA per ml–40 mM imidazole, washed two times with 40 mM imidazole–20 mM Tris HCl (pH 7.5), and finally eluted with 30 μl of 250 mM imidazole–20 mM Tris HCl (pH 7.5). Elutions were performed a second time, and eluates were pooled.

For the in vivo interaction experiments, HF insect cells were infected with a Hisp36^{MAT1}-producing baculovirus either alone or together with the wild-type human p53-producing baculovirus. Cell lysates prepared 48 h later were incubated with anti-p53 monoclonal antibody PAb 421 cross-linked to protein A-Sepharose beads at 4°C for 2 h. The immunoprecipitates were washed four times and resolved by 10% PAGE and Western blotting.

Generation of αpSer33 and αpSer37 antibodies. Phosphopeptides containing phosphorylated Ser-33 [PENNVLS(PO_3) PLPSQAC] or phosphorylated Ser-37 [VLSPLPS(PO_3) QAMDDLC] were chemically synthesized, conjugated to keyhole limpet hemocyanin, and used to immunize rabbits as previously described (29). Polyclonal antibodies were purified from the antiserum by column chromatography with Sepharose CL-4B linked to the immunizing phosphopeptide and, after elution, were passed through a column of Sepharose CL-4B linked to the respective unphosphorylated peptide in order to deplete antibodies reacting with unphosphorylated p53. The purified antibodies recognized their respective phosphorylated but not unphosphorylated peptides in an enzyme-linked immunosorbent assay (data not shown). These antibodies were designated αpSer33 and αpSer37.

Western blots. Kinase assays were performed in the presence or absence of trimeric CAK with 100 ng of GST1-82. Reactions were carried out as described above but with nonradioactive ATP at 100 μM. A 5-μl aliquot of each reaction mixture was removed, and a trace amount of [γ-^{32}P]ATP was added to ensure that GST1-82 had, in fact, been phosphorylated by CAK. Reaction mixtures were then run in parallel sets on an SDS–10% polyacrylamide gel, blotted to nitrocellulose, and probed overnight at 4°C with a polyclonal αpSer33 antibody, a polyclonal αpSer37 antibody, or monoclonal antibody PAb 1801, which recognizes an epitope in the N terminus of p53. After incubation with the appropriate secondary antibodies, the Western blots were visualized by enhanced chemiluminescence (Amersham).

Detection of p53 in cells after DNA damage. RKO and LNCaP human tumor cell lines (80% confluent) were irradiated (7 Gy), and extracts were prepared 2 h later in the presence of phosphatase inhibitors (1 mM Na orthovanadate and 5 mM NaF) and immunoprecipitated with PAb 421–protein A-Sepharose beads as described above. Immunoprecipitates were resolved by SDS-PAGE, followed by transfer to nitrocellulose, and reactivity with an anti-phosphorylated Ser-33 antibody was determined as described above. Blots were then stripped and reprobed with a mixture of anti-p53 monoclonal antibodies PAb 421 and PAb 1801.

RESULTS

CDK7-cyclin H phosphorylates p53 in a p36^{MAT1}-dependent manner. In order to test whether p53 acts as a substrate for phosphorylation by CDK7-cyclin H, bacterium-produced Hisp53 was used in an in vitro kinase assay in the presence or absence of Hisp36^{MAT1}. While CDK7-cyclin H by itself showed a very low level of phosphorylation of Hisp53 (Fig. 1A, lane 5), the addition of Hisp36^{MAT1} increased the phosphorylation of Hisp53 13-fold (Fig. 1A, lane 6). In contrast, GST-CDK2 and cyclin A were both very efficiently phosphorylated by CDK7-cyclin H alone (Fig. 1A, lane 8), and their phosphorylation was not further stimulated by the addition of Hisp36^{MAT1} (Fig. 1A, lane 9).

Trimeric CAK phosphorylates p53 within its first 82 residues, at a site(s) other than the DNA-PK sites. His-tagged deletion mutants of p53 were used to determine the region of p53 phosphorylated by trimeric CAK (see Fig. 2B for a silver-stained gel showing the proteins used). While a mutant protein containing the first 157 residues of p53 (His1-157) was phosphorylated by CAK (Fig. 2A, lane 4), an N-terminally truncated p53 (with a deletion of the first 159 residues, His160-393) was not (Fig. 2A, lane 8), although this same His160-393 protein was capable of being phosphorylated at Ser-315 by CDC2-cyclin B (data not shown; 3). Deletion of the first 30 residues from the N terminus (His31-393) did not significantly reduce the phosphorylation efficiency (Fig. 2A, compare lanes 1 and 2). However, removal of the N-terminal 41 residues decreased phosphorylation efficiency by a factor of 3 to 6 (His42-393; Fig. 2A, lane 3, and data not shown), indicating that a major CAK phosphorylation site on human p53 is located between residues 31 and 42. Consistent with CAK phosphorylating the N termi-

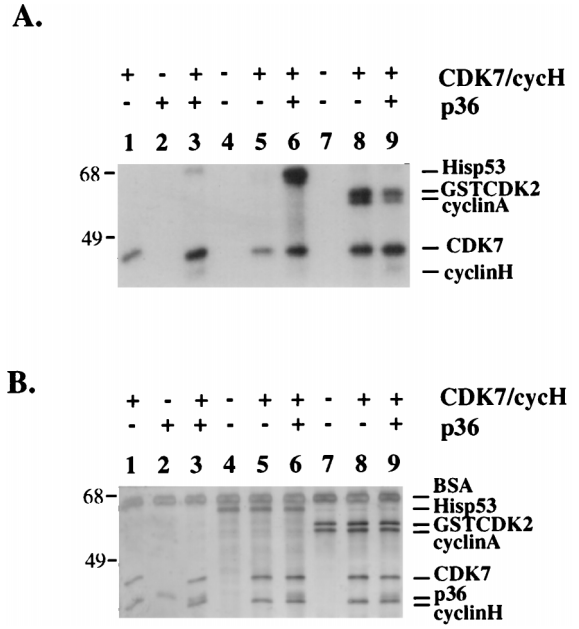

FIG. 1. Phosphorylation of p53 by CDK7-cyclin H (cycH) is strongly stimulated by Hisp36^{MAT1}. Hisp53 (lanes 4 to 6), GST-CDK2–cyclin A (lanes 7 to 9), and no substrate (lanes 1 to 3) were tested for phosphorylation by CDK7-cyclin H (lanes 1, 3, 5, 6, 8, and 9) in the absence (lanes 1, 5, and 8) or the presence (lanes 3, 6, and 9) of Hisp36^{MAT1} in in vitro kinase assays as described in Materials and Methods. Lanes 4 and 7 show that there was no background kinase activity in the absence of CDK7-cyclin H. Reaction mixtures were run on SDS–10% polyacrylamide gels which were silver stained (B) and then dried and exposed for autoradiography (A). The migration of molecular mass markers (in kilodaltons) is shown on the left, and proteins are indicated on the right. CDK7 was autophosphorylated in these reactions (lane 1). The silver-stained gel (B) shows 68-kDa BSA, 54-kDa2 Hisp53, 59-kDa GST-CDK2, 54-kDa cyclin A, 40-kDa CDK7, 34-kDa Hisp36^{MAT1}, and 37-kDa cyclin H. The presence of the His tag alters the mobility of Hisp53 and Hisp36^{MAT1}.

nus of p53, it was observed that GST1-82, containing the first 82 residues of p53, was phosphorylated very efficiently by CDK7-cyclin H in a Hisp36^{MAT1}-dependent manner (Fig. 2C, lanes 2 and 3), while glutathione *S*-transferase (GST) alone was not (data not shown). That His42-393 was phosphorylated by CAK to a lower extent (Fig. 2A, lane 3) while there was no detectable phosphorylation of His160-393 (lane 8), His160-375 (lane 7), or His160-306 (lane 5) indicates the presence of a second, minor CAK phosphorylation site between amino acid residues 42 and 160.

To confirm that CAK predominantly phosphorylated the N terminus of p53, an extensive panel of bacterium-produced and baculovirus-produced mutant proteins of p53 was tested for the ability to be phosphorylated by CAK. The results (Fig. 3) were all consistent with the notion that a major CAK phosphorylation site resides between residues 31 and 42.

There are two potential phosphate acceptor residues in this region, at Ser-33 and Ser-37. Ser-37 is one of the two previously identified DNA-PK phosphorylation sites on p53; the other is at Ser-15 (33). In order to test whether trimeric CAK phosphorylated p53 at the same sites as does DNA-PK, a phosphorylation site mutant protein generated for the DNA-PK sites (Ser-15 and Ser-37) (33), in which the phosphate acceptor sites were mutated to Ala (15A37A), was used as a substrate for CAK. The 15A37A protein was phosphorylated by CAK with the same efficiency as wild-type p53 over background levels (95% the efficiency of the wild type) (Fig. 2E), demonstrating that the CAK phosphorylation site(s) is different from those recognized by DNA-PK. Therefore, the CAK phosphorylation site(s) lies within the first 82 residues of p53, at a site(s) other than the DNA-PK sites.

Trimeric CAK phosphorylates p53 at Ser-33. In order to map the CAK phosphorylation sites on p53, two-dimensional phosphotryptic peptide mapping was performed with Hisp53 after in vitro kinase reactions with CAK. The maps showed one major and one minor phosphorylated peptides with a low mobility in the electrophoretic dimension (*x* axis) and a high mobility in the chromatographic dimension (*y* axis) (Fig. 4A). This pattern is typical of that of the large hydrophobic peptides generated from the N-terminal portion of p53 (41) and corresponds to a single peptide predominantly phosphorylated at a single site. By contrast, p53 phosphorylated by CDC2-cyclin B yielded labeled tryptic peptides with a high mobility in the first dimension and a low mobility in the second, characteristic of C-terminal peptides and consistent with a phosphorylation site location at Ser-315 (Fig. 4B) (3, 41). Multiple peptides were produced as a result of partial trypsin digestion at adjacent Lys residues flanking the Ser-315 site. Because the three tryptic peptides comprising the N terminus of p53 (peptides 1-24, 25-65, and 66-101) all have similar mobilities in both dimensions due to their large size and high degree of hydrophobicity, a comparison between full-length and deletion p53 proteins was required to unambiguously determine which tryptic peptide contained the phosphate acceptor site(s) for CAK. Two-dimensional phosphotryptic mapping was performed with Hisp53, GST1-82, and His31-393. Two-dimensional maps of GST1-82 alone and GST1-82–Hisp53 showed identical migration patterns for their phosphorylated peptides (Fig. 4C and D), indicating that the phosphate acceptor group was either on peptide 1-24 or on peptide 25-65. Maps made with His31-393 consistently showed two peptides with a pattern generally similar to that for full-length Hisp53 (Fig. 4A and E), with the notable addition of another peptide with a higher electrophoretic mobility and a lower chromatographic mobility and whose intensity was variable from experiment to experiment (Fig. 4E). This peptide was likely due to phosphorylation of the His tag, since it was not present in maps made with GST1-82 but was present in maps made with His1-70 (data not shown). Maps obtained when samples from both Hisp53 and His31-393 were run together demonstrated that, in contrast to Hisp53–GST1-82, the labeled peptides did not comigrate (Fig. 4D and F). This result demonstrates that the peptides phosphorylated in Hisp53 and His31-393 were not identical. In fact, compared with the peptide from Hisp53, the major peptide from His31-393 had a lower mobility in the chromatographic dimension and a higher mobility in the electrophoretic dimension, corresponding to a shift down and to the right (Fig. 4F). Although it was difficult to completely resolve the two peptides, they clearly had different migration patterns. These patterns were those which would have been predicted from the differences between peptide 25-65 resulting from tryptic cleavage of full-length Hisp53 and peptide 31-65 generated from the trypsinization of N-terminally-deleted His31-393. Thus, trimeric CAK phosphorylated full-length Hisp53 within residues 25 to 65.

The results of the two-dimensional mapping are consistent with peptide 25-65 being predominantly phosphorylated at a single site and, to a lesser degree, secondarily phosphorylated at another site. There are four potential phosphate acceptor groups in peptide 25-65: Ser-33, Ser-37, Ser-46, and Thr-55. Ser-37 corresponds to the DNA-PK site (33) which, when mutated to Ala, resulted in a protein that was still phosphorylated efficiently by CAK (Fig. 2E). Therefore, the remaining

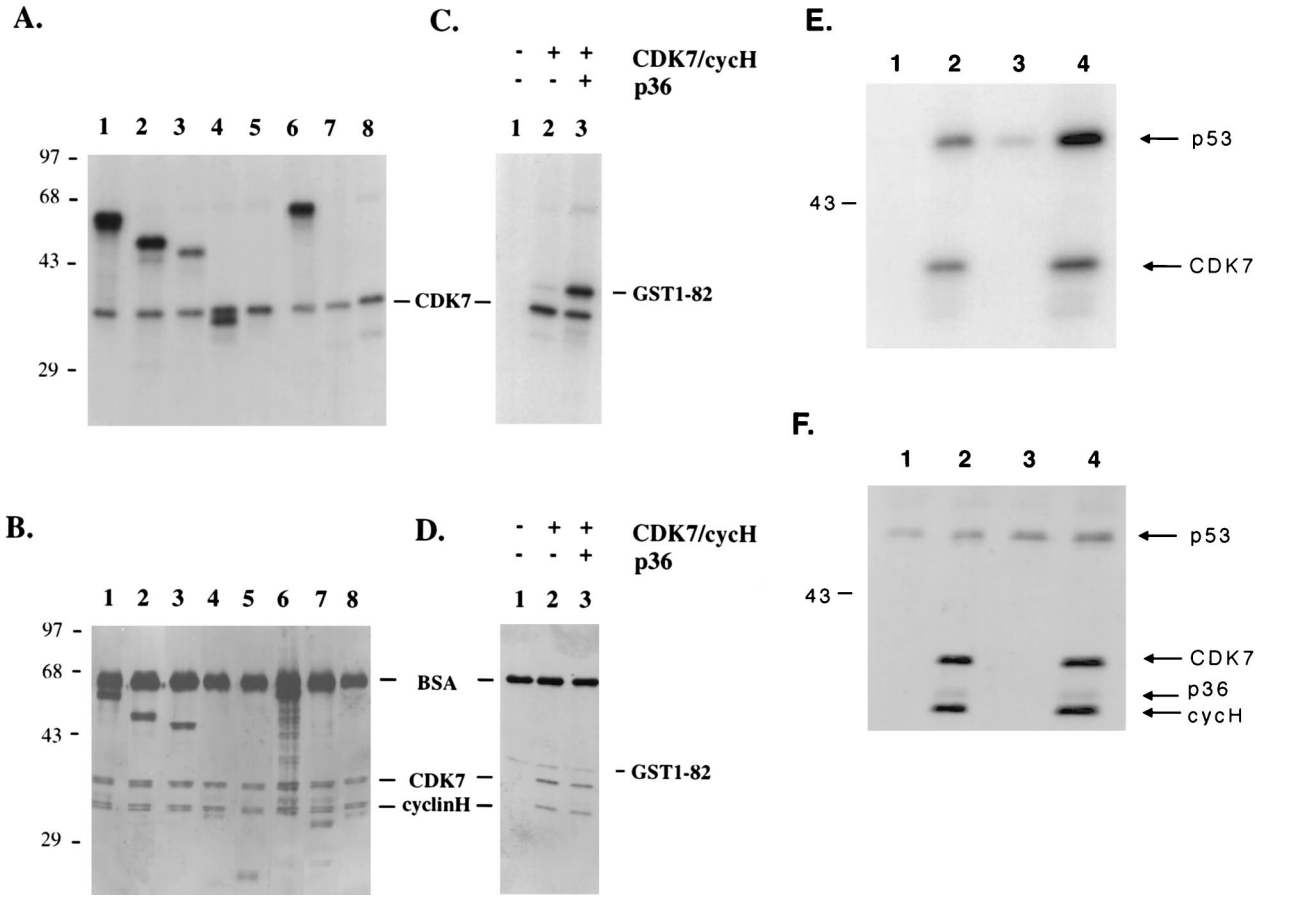

FIG. 2. Trimeric CAK phosphorylates p53 within its N-terminal residues, at a site(s) other than the DNA-PK sites. (A and B) Hisp53 proteins or deletion mutant proteins were used as substrates for phosphorylation by CDK7-cyclin H in the presence of Hisp36^{MAT1} as described in Materials and Methods. Mixtures were subjected to SDS-PAGE and then silver stained (B) and autoradiographed (A) as described in the legend to Fig. 1. Lanes: 1, Hisp53; 2, His31-393; 3, His42-393; 4, His1-157; 5, His160-306; 6, His1-375; 7, His160-375; 8, His160-393. The migration of molecular mass markers (in kilodaltons) is shown on the left. Note that phosphorylation of His1-157 in lanes 4 caused a marked reduction in its electrophoretic mobility. (C and D) CAK phosphorylates p53 within its N-terminal 82 residues. GST1-82 was used as a substrate for phosphorylation by CDK7-cyclin H in the absence (lane 2) or presence (lane 3) of Hisp36^{MAT1} followed by SDS-PAGE, silver staining (D), and autoradiography (C). The migration of GST1-82 is indicated on the right. (E and F) The DNA-PK site mutant p53 protein 15A37A is phosphorylated by CAK over background levels to the same degree as wild-type p53. Either wild-type (lanes 1 and 2) or 15A37A mutant (lanes 3 and 4) p53 proteins were mock phosphorylated (lanes 1 and 3) or phosphorylated by trimeric CAK (lanes 2 and 4) and then subjected to SDS-PAGE followed by silver staining (F) and autoradiography (E).

candidates for phosphorylation by CAK are Ser-33, Ser-46, and Thr-55.

A panel of peptides corresponding to the N terminus of p53 was tested for the ability to be phosphorylated by trimeric CAK. Phosphorylation of the peptides was assayed by phosphocellulose binding methods as described previously (6). From the panel of p53 peptides, the only one that was significantly phosphorylated by CAK above background levels corresponded to residues 27 to 38 (data not shown). This peptide, 33S37S, contained two potential acceptor sites, Ser-33 and Ser-37. Since Ser-37 was not phosphorylated by CAK (Fig. 2E), Ser-33 was predicted to be the major site of phosphorylation on p53 by CAK. Consistent with this conclusion, a peptide with a mutation of the Ser-33 site to Ala was not phosphorylated by CAK over background levels (data not shown).

Further demonstration that Ser-33 on p53 was a CAK phosphorylation site was obtained when we used antibodies directed against phosphorylated epitopes spanning Ser-33 and Ser-37 on p53 (αpSer33 and αpSer37, respectively). We compared the ability of these antibodies to recognize CAK-phosphorylated or unphosphorylated GST1-82 p53. αpSer33 but not αpSer37 recognized p53 only after it had been treated with CAK (Fig. 5). The validity of the negative result obtained with αpSer37 has been repeatedly confirmed in experiments in which we have demonstrated that this antibody efficiently recognizes p53 only after it has been phosphorylated at Ser-37 by DNA-PK (unpublished data). Thus, we have three lines of evidence that CAK phosphorylates p53 at Ser-33: phosphotryptic mapping, phosphorylation of a peptide spanning this site, and recognition by an antibody specific for phosphorylated Ser-33. The assignment of Ser-33 as a major site of phosphorylation by CAK is consistent with the results for the deletion mutants in Fig. 2 in that further deletion between residues 31 and 41 significantly decreased the ability of p53 to act as a substrate for CAK. While there may be other sites that serve as phosphate acceptors for CAK, Ser-33 appears to be the major one on the basis of the predominance of the single spot corresponding to peptide 25-65 in the two-dimensional phosphotryptic maps.

Phosphorylation of tumor-derived mutant p53 proteins by CAK. In order to test whether tumor-derived mutant p53 proteins would be phosphorylated by CAK, baculovirus-produced Val143Ala, Arg248Trp, Arg249Ser, and Arg273His mutant proteins (see reference 19) were used in in vitro kinase assays.

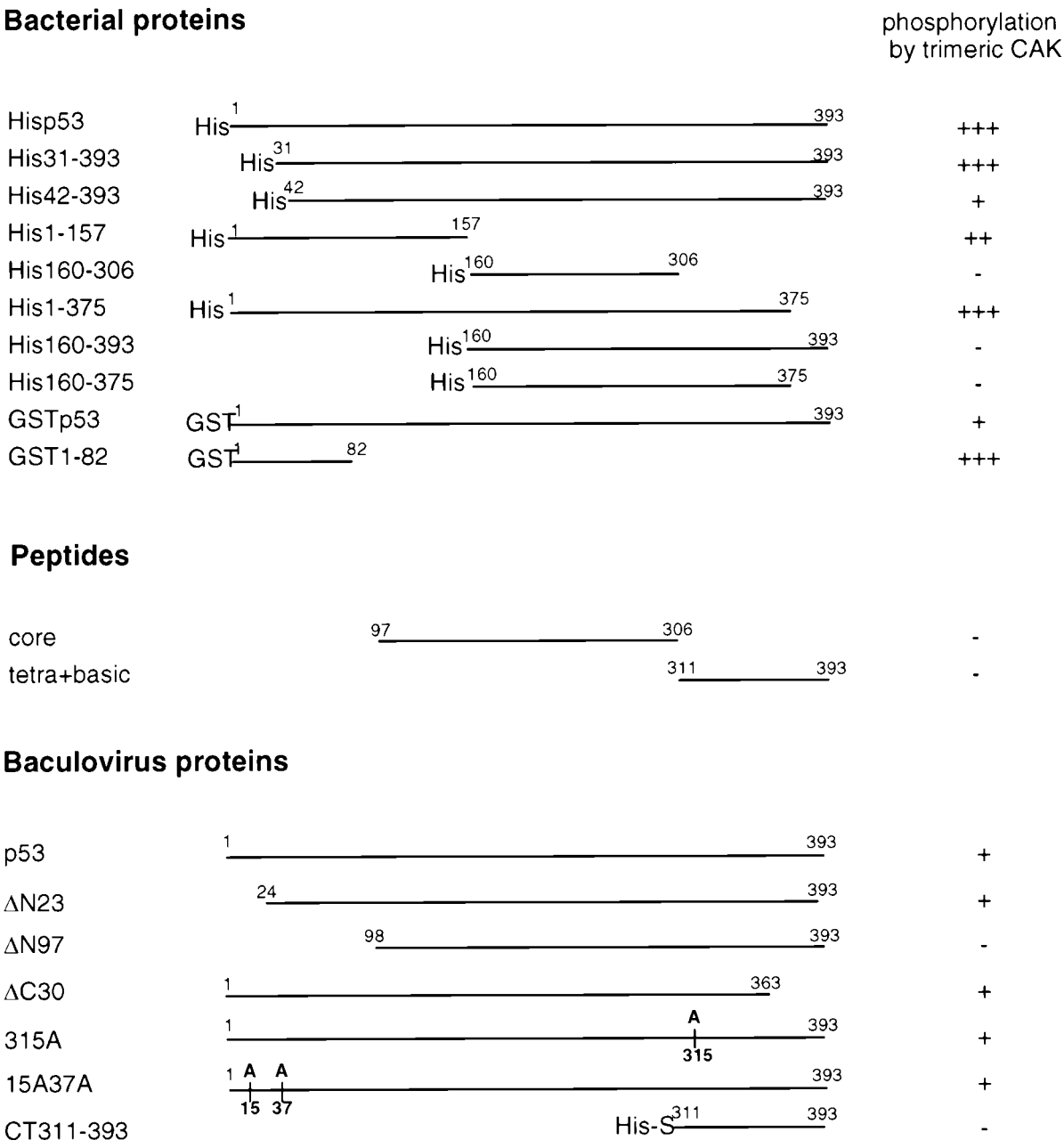

FIG. 3. Summary of bacterium- and baculovirus-produced proteins tested for the ability to be phosphorylated by trimeric CAK. The deletion mutant versions of p53 used in vitro kinase assays as described in Materials and Methods are represented schematically. The ability to be phosphorylated by CAK was determined by the degree of [γ-^{32}P]ATP incorporation in the presence of CAK relative to that in the absence of CAK.

Each of these proteins was phosphorylated by CAK at levels comparable to wild-type p53 (Fig. 6A). Baculovirus-expressed Arg175His was also significantly phosphorylated by CAK (data not shown). These results were confirmed with bacterium-expressed GST fusion proteins (GST143, GST175, and GST273); each of these was also phosphorylated by CAK (data not shown). Therefore, tumor-derived mutant p53 proteins also serve as substrates for CAK.

p36^{MAT1} and p53 physically associate in vitro. We were interested in testing whether p53 directly interacts with p36^{MAT1}, since protein-protein interactions appear critical to p36^{MAT1} functioning in its role as an assembly factor, facilitating association between CDK7 and cyclin H. ^{35}S-labeled full-length and deletion mutant p36^{MAT1} proteins were produced in rabbit reticulocyte lysates, as was the p53-interacting protein MDM2. These proteins were then used in in vitro binding assays with Hisp53. As previously demonstrated, MDM2 was specifically coimmunoprecipitated by anti-p53 antibodies in the presence of Hisp53 but not in the absence of Hisp53 (Fig. 7A, lanes 4 and 8). Similarly, full-length p36^{MAT1} protein was also coimmunoprecipitated by antibodies recognizing p53 specifically in the presence of Hisp53 (Fig. 7A, lanes 1 and 5). By contrast, deletion mutant p36^{MAT1} proteins were not capable of associating with Hisp53 (Fig. 7A, lanes 2, 3, 6, and 7). The

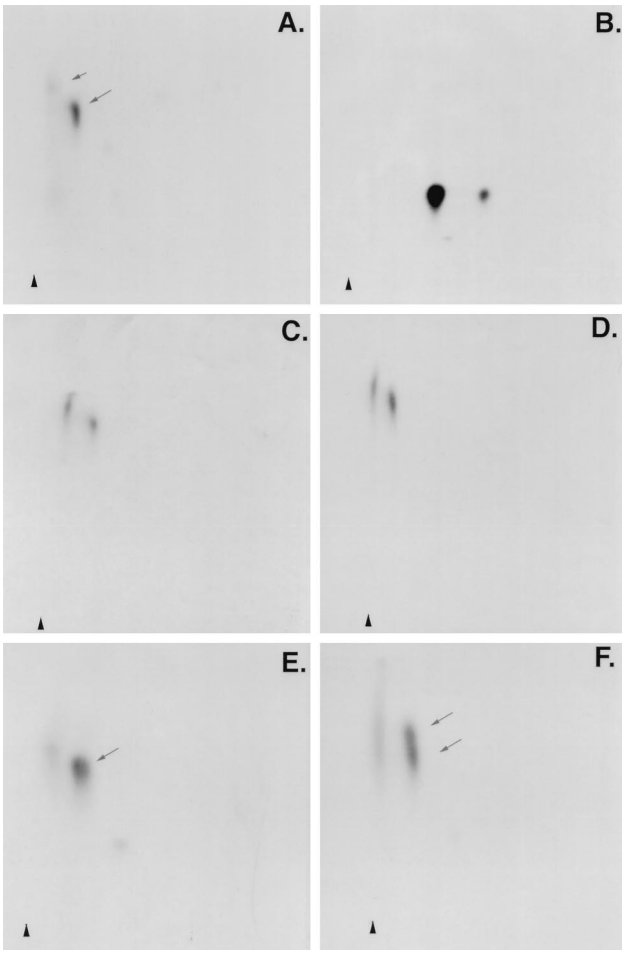

FIG. 4. Mapping of the trimeric CAK phosphorylation site on p53. (A) Two-dimensional phosphotryptic peptide maps of Hisp53 phosphorylated by trimeric CAK in vitro were generated. The longer arrow indicates a major singly phosphorylated peptide, and the shorter arrow shows a minor peptide containing two phosphate groups. (B) Map of baculovirus-expressed p53 after phosphorylation by CDC2-cyclin B in vitro. (C) Map of CAK-phosphorylated GST1-82 alone. (D) Map of CAK-phosphorylated GST1-82 run with tryptic peptides of CAK-phosphorylated Hisp53. (E) Map of CAK-phosphorylated His31-393 alone. (F) Map of CAK-phosphorylated His31-393 run with CAK-phosphorylated Hisp53 tryptic peptides. The arrows in panels E and F indicate the lower mobility of the 31-65 peptide from His31-393 than of the 25-65 peptide from Hisp53. Arrowheads represent the origin.

binding of full-length p36^{MAT1} corresponded to 4% of the input labeled protein (Fig. 7A, lanes 1 and 10).

In the reciprocal experiment, full-length and truncated versions of p53 were in vitro translated and labeled with ^{35}S. A low but specific level of full-length p53 was associated with Ni-NTA–agarose beads recognizing the His tag on Hisp36^{MAT1} in the presence of Hisp36 but not with Ni beads alone (Fig. 7B, lanes 1 and 4). Notably, a significant amount of a p53 protein with a truncation in the N terminus (95-393) associated with Hisp36 (Fig. 7B, lanes 2 and 5). Since this protein contained a deletion of the first 94 residues of p53, including the domain required for CAK phosphorylation (residues 1 to 82) (Fig. 2 and 4), p36^{MAT1} binding of the N terminus of p53 was not strictly required for trimeric CAK to phosphorylate p53. Therefore, p53 and p36^{MAT1} associate in vitro and the N terminus of p53 is not necessary for this interaction.

p53 binds p36^{MAT1} and is phosphorylated at Ser-33 in cells. To bring the in vitro experiments described above into a cellular context, we used two approaches. First we sought to demonstrate that p36 can interact with p53 in vivo. This was accomplished by coinfection of insect cells with a recombinant baculovirus expressing Hisp36 alone or together with one expressing wild-type p53. After incubation of infected cell extracts with anti-p53 antibody, it was clear that significantly more Hisp36 was immunoprecipitated in the presence of p53 than in its absence (Fig. 8A, compare lanes 5 and 6). It should be noted, however, that the interaction between p53 and p36 was rather weak both in vitro and in vivo. Moreover, we cannot assess whether these two polypeptides interact directly in mammalian cells.

Our second approach was to use an anti-phosphorylated Ser-33 antibody to determine whether p53 is phosphorylated at Ser-33 in cells. Indeed, this antibody recognized p53 stabilized by gamma irradiation (Fig. 8B) as well as by UV irradiation (data not shown) in at least two cell lines, RKO and LNCaP, both of which contain inducible wild-type p53. Although these experiments did not address whether p53 is phosphorylated at Ser-33 by CAK in these or other cells or in fact whether phosphorylation at this site is actually induced by irradiation, the data obtained in vivo nevertheless suggest that our in vitro results are physiologically relevant.

DISCUSSION

In response to DNA damage, p53 mediates cell cycle arrest by transcriptional activation of the *p21* gene, which encodes a CDK inhibitor (for a review, see references 20 and 30). In contrast to the detailed mechanism which has been elucidated for the events downstream of p53, relatively little is known about the upstream events by which DNA strand breaks lead to upregulation of p53 levels and activity. The p53 protein is phosphorylated by a wide variety of protein kinases both at its N terminus, by casein kinase I, DNA-PK, JNK, and MAP kinases, and at its C terminus, by CDKs, PKC, and casein kinase II (see reference 40 for a review). In this study, we have shown that p53 can also be phosphorylated in vitro by CAK and that this phosphorylation is dependent on the p36^{MAT1} subunit of CAK (Fig. 1). Trimeric CAK phosphorylates p53 at its N terminus, and the major site of phosphorylation is residue Ser-33, a site which we determined is also phosphorylated in vivo. While this site is not precisely conserved in evolution, there is a Ser-34 in murine p53. This residue corresponds to the previously identified site of phosphorylation of the UV-induced kinase JNK in murine p53. UV irradiation leads to increased phosphorylation of p53 at this site (42). Interestingly, the kinase activity of TFIIH-associated CAK was reported to be reduced after UV irradiation (1). The potential interplay between these kinases to phosphorylate the N terminus of p53,

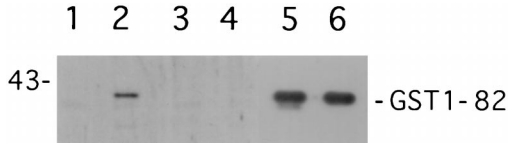

FIG. 5. Trimeric CAK phosphorylates p53 at Ser-33. GST-1-82 was incubated in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of trimeric CAK. After transfer to nitrocellulose, blots were incubated with a polyclonal αpSer33 antibody (lanes 1 and 2), a polyclonal αpSer37 antibody (lanes 3 and 4), or monoclonal antibody PAb 1801 (lanes 5 and 6), which recognizes residues 46 to 55 in the N terminus of p53. Proteins were visualized with enhanced chemiluminescence. CAK-phosphorylated GST1-82 was specifically recognized by the αpSer33 antibody. Kilodaltons are given on the left.

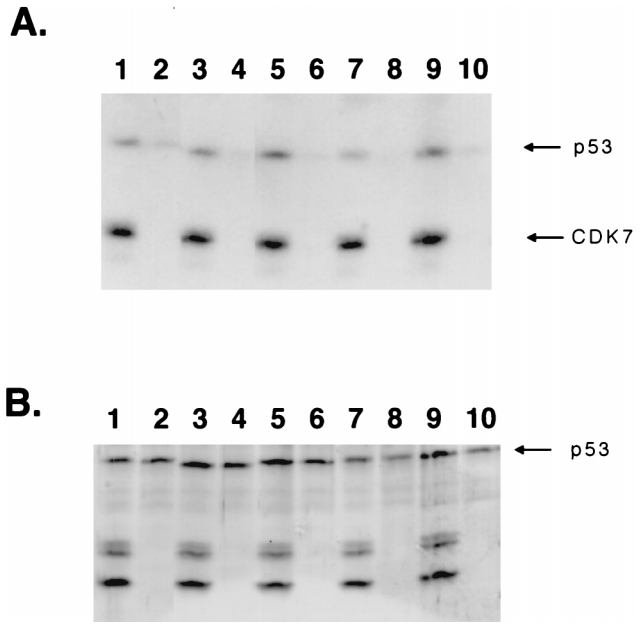

FIG. 6. Phosphorylation of tumor-derived mutant p53 proteins by CAK. (A) In vitro kinase assay. Wild-type p53 (lanes 1 and 2) and Val143Ala (lanes 3 and 4), Arg248Trp (lanes 5 and 6), Arg249Ser (lanes 7 and 8), and Arg273His (lanes 9 and 10) p53 mutant proteins expressed from baculovirus-infected insect cells were incubated in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of trimeric CAK. (B) Silver-stained gel. Lanes are as described in panel A.

particularly in response to UV irradiation, remains to be addressed.

Phosphorylation of p53 at its N terminus has been shown to affect p53 transactivation and suppression of transformation in a manner in which multiple substitutions at phosphorylation acceptor sites have additive phenotypic effects (39, 43). Thus, it is not surprising that singly mutating Ser-33 to alanine did not affect the ability of p53 to activate the transcription of reporter genes or to suppress growth (64). Thus, the functional relevance of the phosphorylation of p53 at its N terminus by CAK remains to be determined. It is possible that phosphorylation by CAK affects the association of p53 with other proteins interacting with the N terminus, such as MDM2 or the TAFs. Experiments are currently under way to test this hypothesis.

We have also demonstrated that several tumor-derived mutant p53 proteins can be phosphorylated efficiently by CAK. Two types of mutants have been classified on the basis of their contribution to DNA binding (7). While some mutants have substitutions at residues that directly contact the DNA, such as residues 248 and 273, other mutations affect the conformation of the entire DNA binding region, such as those at residues 175 and 249. Our data show that both contact and conformational mutants are phosphorylated by CAK (Fig. 6).

Dual specificity of CAK. The observation that p53 requires p36^{MAT1} to be efficiently phosphorylated by CDK7-cyclin H but that GST-CDK2 does not raises the intriguing possibility that CAK may have a dual substrate specificity determined by the presence of the p36^{MAT1} protein. CAK is known to phosphorylate both the T loop of CDKs and the CTD of RNA polymerase II, although there is no obvious sequence similarity in their respective phosphorylation sites, suggesting that CAK may be able to recognize and phosphorylate multiple targets. The presence of p36^{MAT1} was recently shown to determine the substrate specificity of CDK7-cyclin H in that the presence of p36^{MAT1} favored the phosphorylation of a CTD substrate over a CDK2 substrate (70). It is interesting to compare the sequences in the CTD (YSPTSPS) and the region near Ser-33 of p53 (VLSPLPSQ) because phosphorylation of both is strongly stimulated by p36^{MAT1}. Both sequences have multiple Ser residues adjacent to Pro residues, and these similarities might be important in determining the substrate specificity of CDK7-cyclin H.

p36^{MAT1} may serve to direct CAK phosphorylation to additional substrates beyond the scope of the CDK T loops, which may influence the apparent paradox that while CAK is believed to activate CDKs to drive the cell cycle, its activity is constant throughout the cell cycle (5, 47, 62). An additional level of regulation of CAK specificity operates by its association with TFIIH. CAK in the context of TFIIH can phosphorylate TFIIE and TFIIF, but free CAK cannot (69); furthermore, TFIIH has a strong preference for the CTD over CDK2 as a substrate, relative to free CAK (69). It will therefore be

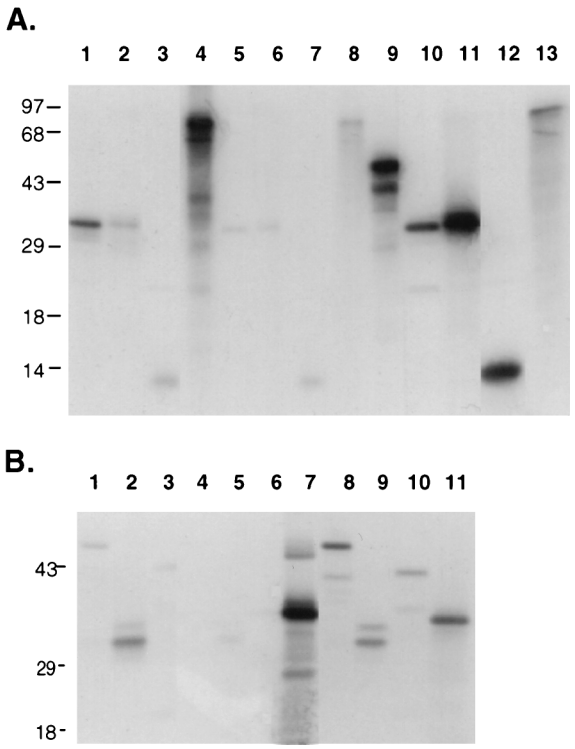

FIG. 7. p36^{MAT1} and p53 associate in vitro. (A) [^{35}S]methionine-labeled full-length and deletion mutant p36^{MAT1} proteins were produced in rabbit reticulocyte lysates and used in in vitro binding assays with Hisp53 as described in Materials and Methods. Proteins were coimmunoprecipitated by anti-p53 antibodies in the presence of Hisp53 (lanes 1 to 4) or in the absence of Hisp53 (lanes 5 to 9). The proteins used were full-length p36^{MAT1} (lanes 1, 5, and 10), His1-253 (lanes 2, 6, and 11), His205-309 (lanes 3, 7, and 12), MDM2 (lanes 4, 8, and 13), and p53 (lane 9). Ten percent of the input in vitro-translated material used in each precipitation was loaded in lanes 10 to 13. Note that the in vitro-synthesized His1-253 contains 35 extra amino acid residues at its N terminus (see Materials and Methods) and therefore migrates in a manner similar to that of the full-length p36^{MAT1} protein. (B) The N-terminal region of p53 is not required for the association of p53 with p36^{MAT1} in vitro. [^{35}S]methionine-labeled full-length and deletion mutant p53 proteins were produced in rabbit reticulocyte lysates and used in in vitro binding assays with Hisp36 as described in Materials and Methods. Proteins were coprecipitated with Ni-NTA–agarose beads in the presence (lanes 1 to 3) or the absence (lanes 4 to 7) of Hisp36. The proteins used were p53 (lanes 1, 4, and 8), 95-393 (lanes 2, 5, and 9), 1-302 (lanes 3, 6, and 10), and Hisp36 (lanes 7 and 11). Ten percent of the input in vitro-translated material used in each precipitation was loaded in lanes 8 to 11. Lanes 1 to 7 were exposed 14-fold longer than lanes 8 to 11. Kilodaltons are given on the left.

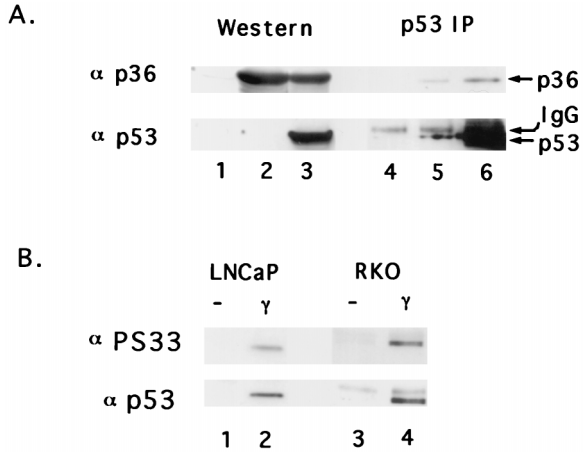

FIG. 8. In vivo interaction of p53 with p36^{MAT1} and phosphorylation of p53 at Ser-33. (A) p36^{MAT1} binds p53 in a cellular environment. HF insect cells were mock infected (lanes 1 and 4), infected with Hisp36-producing baculovirus alone (lanes 2 and 5), or coinfected with His-p36- and p53-producing baculoviruses (lanes 3 and 6). Cells were lysed, and immunoprecipitation was performed with the anti-p53 antibody PAb 421 cross-linked to protein A-Sepharose beads. Immunoprecipitated proteins (p53 IP) (lanes 4 to 6) and 20% of the input material (lanes 1 to 3) were then analyzed by Western blotting with anti-p36 antiserum (αp36) or anti-p53 antibodies PAb 421 and PAb 1801 (αp53). IgG, immunoglobulin G. (B) p53 is phosphorylated at Ser-33 in vivo. LNCaP cells (lanes 1 and 2) and RKO cells (lanes 3 and 4) were gamma irradiated (γ), and cell lysates were prepared 2 and 3 h later, respectively. p53 was immunoprecipitated from the lysates and detected by Western blotting with anti-phosphorylated Ser-33 antiserum (αPS33). The blot was then stripped and reprobed with anti-p53 antibodies PAb 421 and PAb 1801 (αp53).

interesting to examine the ability of TFIIH-associated CAK to phosphorylate p53.

p53 and transcription and repair. The association of p36^{MAT1} with p53 provides yet another example of a TFIIH component which interacts with p53, in addition to XPB, XPD, and p62 (34, 65, 66). p53 inhibits the helicase activity of XPB and XPD, presumably by its strand-reannealing properties (34, 66), but whether TFIIH association affects p53 function is not yet known. It may be that these multiple associations between TFIIH and p53 serve to colocalize p53 at a site of DNA repair or transcriptional initiation. Additionally, they may serve to bring p53 in proximity to CAK such that it can then be phosphorylated by CAK, potentially coupling the regulation of p53 function with DNA damage or transcription. It will therefore be of great interest to determine whether phosphorylation by CAK affects the transcriptional activity of p53 or its ability to interact with other proteins, thus potentially regulating the tumor suppressor functions of p53.

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