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The CDK_7-cycH-p36 Complex of Transcription Factor IIH Phosphorylates p53, Enhancing Its Sequence-Specific DNA Binding Activity In Vitro

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Phosphorylation is believed to be one of the mechanisms by which p53 becomes activated or stabilized in response to cellular stress. Previously, p53 was shown to interact with three components of transcription factor IIH (TFIIH): excision repair cross-complementing types 2 and 3 (ERCC2 and ERCC3) and p62. This communication demonstrates that p53 is phosphorylated by the TFIIH-associated kinase in vitro. The phosphorylation was found to be catalyzed by the highly purified kinase components of TFIIH, the CDK_7-cycH-p36 trimeric complex. The phosphorylation sites were mapped to the C-terminal amino acids located between residues 311 and 393. Serines 371, 376, 378, and 392 may be the potential sites for this kinase. Phosphorylation of p53 by this kinase complex enhanced the ability of p53 to bind to the sequence-specific p53-responsive DNA element as shown by gel mobility shift assays. These results suggest that the CDK_7-cycH-p36 trimeric complex of TFIIH may play a role in regulating p53 functions in cells.

The tumor suppression function of p53 is attributed primarily to its regulatory roles in cell cycle and apoptosis. In response to cellular stress such as hypoxia or DNA damage, p53 either functions as a checkpoint to arrest cells at the G_1 phase of cell cycle or triggers the apoptotic apparatus to induce programmed cell death (for reviews, see references 24, 37, and 40), thus preventing the proliferation of cells harboring damaged genes.

The p53 protein is a nuclear phosphoprotein that acts as a transcriptional activator (17, 62) with a sequence-specific DNA-binding activity (10, 17, 58, 62). The transcriptional activity of p53 represents one of the mechanisms by which p53 regulates cell growth and death. Most of the p53 mutations, in a wide variety of human cancers, are found in this central sequence-specific DNA-binding domain (41). The MDM2 protein, a negative regulator of p53 (54), binds to the N-terminal transactivation domain of p53 and thus limits its ability to transactivate the expression of p53-responsive genes (8) and to induce a G_1 arrest (9). Interestingly, RNA synthesis from the *MDM2* gene itself is stimulated by p53 after DNA damage, thus forming an autoregulatory feedback loop (59, 76). An increasing number of p53 target genes have been identified, and some of them have been implicated in the p53 downstream pathway regulating the cell cycle and apoptosis (24, 37). For example, $p21^{waf1/cip1}$, a broad inhibitor of CDK_2/CDC_2 kinases (78), is activated by p53 in response to DNA damage (14, 15). In addition, the induction of p21 has been shown to be at least in part responsible for G_1 arrest (14, 15). The *Bax-1* gene, which promotes apoptosis (57), is also upregulated by p53 in certain cell types (53).

Although it is known that p53 is activated and stabilized by damaged DNA (35), hypoxia (25), or a lower level of ribonucleoside triphosphates (43), it remains unclear how this happens. An attractive hypothesis is that it may occur through posttranslational phosphorylation of p53 (31). p53 is phosphorylated at multiple sites in vivo, which have been localized to the N-terminal and C-terminal regions of the protein (48, 65). Also, p53 can be phosphorylated in vitro by a number of protein kinases including members of the CDK_2 family (5, 75), CKI (52), CKII (30), PKC (3), DNAPK (18), mitogen-activated protein kinase (49), Raf1 (33), and the c-Jun kinase, JNK1 (50). The recombinant p53 protein purified from *Escherichia coli*- or baculovirus-infected cells fails to bind to the p53RE (31). Phosphorylation of p53 with some of these kinases enhances the ability of p53 to specifically bind to the P53RE (3, 30, 52, 75). Thus, this could represent a mechanism to activate p53 transcription after a stress signal. However, a more definitive physiological correlation between these kinases and the p53 functions has yet to be established.

In an attempt to address this issue, the ability of the basal transcription factor, TFIIH (22), to phosphorylate p53 was studied, for the following reasons. First, TFIIH is actively involved in DNA repair and probably regulation of the cell cycle. p53 was reported to bind to damaged DNA in vitro (39) and to possess a 3′-5′ exonuclease activity (55), and thus it could play a role in facilitating DNA repair (39, 55). TFIIH, which is a multisubunit protein complex consisting of ERCC2 (66), ERCC3 (67), p62 (19), CDK_7 (23, 60, 70), cycH (21), p36 (MAT1) (20, 38, 72), and possibly other polypeptides, contains two intrinsic enzymatic activities: helicase (66, 67) and kinase activities (64, 68, 69). ERCC2 (XPB) and ERCC3 (XPD) are the helicases involved in NER (12, 16). The CDK_7-cycH-p36 complex phosphorylates the CDK_2 family members in vitro (20, 38, 72) and hence is called CAK (for the CDK_2-activating kinase) (71). While a physiological role for the CAK complex has not yet been definitively established, it is clear that components of TFIIH have dual functions of NER and cell cycle regulation in addition to being essential for transcription initiation (12). Second, p53 was found to bind to three large

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components (ERCC2, ERCC3, and p62) of TFIIH in vitro (73, 77) and to inhibit the helicase activity of TFIIH (73). Recently, the two helicase subunits of TFIIH were shown to be important elements required for p53-induced apoptosis in vivo (74). These studies therefore point to a functional link between p53 and TFIIH and to the testable hypothesis that the TFIIH-associated kinase phosphorylates p53 and alters p53 activity.

This paper demonstrates that the CAK complex of TFIIH phosphorylates p53 in vitro. This phosphorylation is significantly stimulated in the presence of p36, a CDK_7-cycH assembly factor (20, 38, 72), and p36 binds to p53, probably mediating this interaction. The phosphorylation occurs in the carboxy-terminal domain of p53, and serines 371, 376, 378, and 392 are the possible phosphorylation sites for this kinase. The phosphorylation of p53 by the CAK-p36 complex enhances the sequence-specific DNA-binding activity of p53 in vitro. A model for how p53 is activated by the TFIIH-associated kinase in response to cellular stress is discussed in this paper.

MATERIALS AND METHODS

Abbreviations. aa, amino acid; ATM, ataxia-telangiectasia mutated; CAK, CDK_2-activating kinase; CKI, casein kinase I; CKII, casein kinase II; DNAPK, DNA-dependent protein kinase; DTT, dithiothreitol; ERCC2 and ERCC3, excision repair cross complementing 2 and 3; GST, glutathione-*S*-transferase; MDM2, the murine double minute 2 oncogene; NER, nucleotide excision repair; NP-40, Nonidet P-40; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; p53RE, p53-responsive DNA element; SCID, severe combined immune deficiency; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFIIH, transcription factor IIH; wt, wild type; XPD and XPB, xeroderma pigmentosum D and B.

Reagents, buffers, and cells. The PKC inhibitor and casein were purchased from Sigma. PKC and CKII were purchased from Boehringer Mannheim. CKI was purchased from Promega. The p53 peptides were synthesized by the Synthetic Core Facility in our department. Anti-CDK_7 and anti-cycH antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-ERCC3 monoclonal antibodies were generously provided by Danny Reinberg (University of Medicine and Dentistry of New Jersey, Piscataway, N.J.). Lysis buffer is composed of 50 mM Tris-HCl (pH 8.0), 0.5% NP-40, 5 mM EDTA, 150 mM NaCl, and 1 mM PMSF. SNNTE is composed of 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% NP40, 500 mM NaCl, and 5% sucrose. Buffer C 100 (BC100) contains 20 mM Tris-HCl (pH 7.9), 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 4 mM $MgCl_2$, 0.2 mM PMSF, 1 mM DTT, and 0.25 μg of pepstatin A per ml. HeLa (a human cervical tumor cell line) and H1299 (a human lung small cell carcinoma cell line) were maintained in Dulbecco-modified Eagle medium (GIBCO-BRL) supplemented with 10% fetal bovine serum. SF9 insect cells were cultured in Grace's insect medium supplemented with antibiotic-antimycotic (GIBCO-BRL).

TFIIH purification. TFIIH was partially purified from HeLa nuclear extracts as previously described (22). HeLa cell nuclear extracts prepared from approximately 10^{10} cells (11) were used as starting materials (0.6 g of protein in 60 ml). The sample was fractionated through phosphocellulose, and proteins were collected by stepwise washing with BC100, BC300 (0.3 M KCl), BC500 (0.5 M KCl) and BC1000 (1.0 M KCl). After dialysis against BC100, the 0.5 M wash (60 mg) was loaded onto a DEAE-Sephacel column. The proteins were washed with 0.5 M KCl-containing buffer C. The 0.5 M wash (5 mg) from the DEAE-Sephacel column was loaded onto a Mono S column (Pharmacia HR5/5) and eluted with a 20-ml linear gradient of KCl (0.1 to 0.7 M). The pool (1 mg) of fractions containing TFIIH was dialyzed against buffer C with 1.2 M ammonium sulfate and loaded onto a phenyl-Superose column (HR5/5) in the SMART system (Pharmacia). The proteins were eluted in a 12-ml linear gradient of ammonium sulfate (1.2 to 0.0 M). The TFIIH-containing fractions (80 μg) were dialyzed against BC100, concentrated through a μ-Mono S column in the SMART system, and stored at −80°C for assays.

The ERCC3-associated TFIIH was prepared by immunoprecipitation with anti-ERCC3 antibodies, as described previously (69). A 400-μl volume of the TFIIH-containing 0.5 M fraction from the phosphocellulose column (see above) was incubated with 40 μl of protein A-Sepharose 12 and 100 ng of anti-ERCC3 antibodies at 4°C for 6 h. The beads were washed with lysis buffer once, SNNTE twice, and BC100 twice. The bead-bound TFIIH was analyzed by SDS-PAGE, followed by Western blotting with antibodies specifically against ERCC3, p62, CDK_7, and cycH, and used for kinase assays.

The GST-CDK_7 protein kinase was activated in vitro as described below. The GST-CDK_7-bound beads were extensively washed with buffer C containing 400 mM KCl. The beads (100 μl) were then incubated at room temperature for 1 h with 700 μl of the TFIIH-containing 0.3 M salt fraction derived from the DEAE-cellulose column. After extensive washing with BC100, the beads were assayed for CDK_7 kinase activity.

Purification of p53 and its mutant 5SA. The p53 mutant 5SA with five residues replaced with alanines at Ser371, Ser376, Thr377, Ser378, and Ser392 was generated in PRC/CMVp53 by a previously described method (42). This mutant was confirmed by sequencing and then subcloned into a baculovirus vector (GIBCO-BRL) for overexpression in SF9 cells. p53 and its mutant 5SA were purified from baculovirus-infected Sf9 insect cells by the previously described method (54, 76).

RNA polymerase II purification. The human RNA polymerase II was purified from HeLa cells as described previously (45).

Affinity purification of the CDK_7-cycH-p36 complexes from Sf9 insect cells. Sf9 insect cells were either triply infected by the Ha-tagged CDK_7-, cycH-, and p36-containing baculoviruses or coinfected by the Ha-CDK_7- and cycH-containing viruses. The cells were then harvested 48 h after infection, and lysed in lysis buffer for protein purification. After the mixture was centrifuged at 10,000 rpm for 10 min, the supernatant was transferred to a fresh tube. A 1.0-ml volume of the supernatant was precleaned with protein A-Sepharose 12B (100 μl) and incubated with 12CA5 beads (200 μl) at 4°C for 6 h. The beads were then washed with lysis buffer twice, SNNTE twice, and BC100 twice. The bound protein kinases were eluted with 0.5 mg of Ha peptides per ml in BC100 into four fractions. The eluted proteins were dialyzed against BC100 and analyzed by SDS-PAGE, silver staining, and Western blotting. The purified proteins were stored at −80°C.

GST fusion protein expression and purification. The GST-CDK_2 and GST-CDK_7 constructs were generated as previously described (21). The fusion proteins were expressed in and purified from *E. coli* as described in the Pharmacia manual.

Western blotting. The purified CDK_7-cycH-p36 trimer and CDK_7-cycH dimer complexes were subjected to SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, and the membrane was probed with primary antibody 12CA5 for 2 h. After being washed three times with phosphate-buffered saline (pH 7.5) containing 0.05% Tween 20, the membrane was immunoblotted with horseradish peroxidase-coupled secondary antibodies for 30 min. Following three washes with the same buffer, the proteins were detected with enhanced chemiluminescence reagents (Amersham). The membrane was then reprobed with anti-CDK_7 and anti-cycH antibodies.

Immunoprecipitation. Immunoprecipitation was conducted by a previously described approach (29). HeLa and H1299 cell extracts (5×10^7 cells) were prepared with lysis buffer containing phosphatase inhibitors (50 mM Na_2MoO_4, 20 mM NaF, 10 mM Na_3VO_4). A 500-μl volume of the cell extracts (450 μg of protein) was incubated with 40 μl of protein A beads (50% slurry) and anti-ERCC3 and anti-cycH antibodies at 4°C for 6 h. The beads were washed with the same lysis buffer twice, SNNTE twice, and the kinase buffer twice (20 mM Tris-HCl [pH 7.4], 10 mM $MgCl_2$, 1 mM DTT, and the same phosphatase inhibitors as above) before being resuspended in the kinase buffer to a final volume of 40 μl. A 10-μl volume of this bead suspension was used for $GCDK_2$ and p53 phosphorylation as described below.

In vitro CDK_7 kinase assay. The CDK_7 kinase assay was carried out by a previously described method (21). A 20-μl volume of reaction mixture contained 20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, 20 μM of [γ-^{32}P]ATP, substrates (0.1 μg of $GCDK_2$, 40 ng of p53, 80 ng of RNA polymerase II, or 100 ng of casein), and either 20 ng of the CDK_7 kinase complexes (trimeric or dimeric) or 10 μl of the aforementioned immunoprecipitated kinases. The mixture was incubated at 30°C for 60 min, and the reaction was stopped by adding the protein-loading buffer (1% SDS, 10 mM β-mercaptoethanol, 0.02% bromophenol blue). The phosphorylated proteins were subject to electrophoresis on an SDS–10% polyacrylamide gel and detected by autoradiography.

In vitro PKC assay. PKC assays were performed as previously described (3). The reaction mixture (20 μl) contained 20 mM Tris-HCl (pH 7.0), 1 mM $CaCl_2$, 8 mM $MgCl_2$, 3 μg of phosphatidylserine per ml, 0.3 μg of diacylglycerol per ml, 20 μM [γ-^{32}P]ATP, 80 μU of PKC, and substrates as indicated in figure legends. The reaction mixture was incubated at 30°C for 60 min, and the reaction was stopped by addition of the protein-loading buffer. The phosphorylated proteins were analyzed by SDS-PAGE and detected by autoradiography.

In vitro CKII and CKI assays. CKII and CKI assays were carried out as previously described (32, 53), with p53 and its mutants as substrates. We used 60 ng of p53, 0.1 mU of CKII, and 0.9 U of CKI in the experiments.

Protein-protein interaction assays with glutathione-Sepharose 12B beads. The fusion proteins were overexpressed in *E. coli* and purified through a glutathione-Sepharose 12B column. Protein-protein association assays were conducted as previously described (46) with the fusion protein-containing beads. The purified CDK_7-cycH-p36 trimeric or CDK_7-cycH dimeric complexes were incubated with the GST-p53 and GST beads (50% slurry), respectively, with approximately 300 ng of fusion proteins. After a 1-h incubation at room temperature, the mixtures were washed three times in buffer B (50 mM Tris-HCl [pH 7.9], 10% glycerol, 0.1 mM EDTA, 0.2 mM PMSF, 1 mM DTT, 0.1% NP-40) containing 200 mM NaCl. The bound proteins were analyzed on an SDS–10% polyacrylamide gel. The bound kinase proteins were detected by Western blotting with anti-p36 and 12CA5 monoclonal antibodies.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assay was based on a published method (58). The protein components, as indicated in

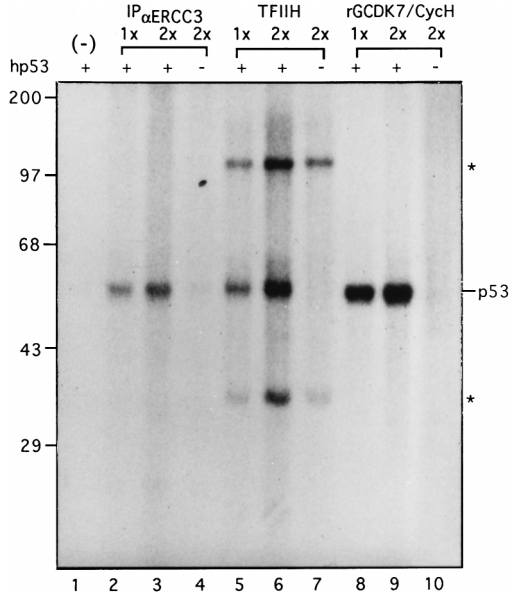

FIG. 1. p53 is phosphorylated by the TFIIH-associated kinase. The proteins used in the experiment are indicated at the top. Lanes (+) contain 40 ng of p53. IP$_{\alpha ERCC3}$ indicates that the TFIIH protein was immunoprecipitated by anti-ERCC3 monoclonal antibodies (1× and 2× represent 5 and 10 μl of the immunoprecipitated proteins, respectively). We used 100 ng (1×) and 200 ng (2×) of the purified TFIIH. rGCDK7/CycH denotes the CDK_7 kinase activated by incubating GST-CDK_7-beads with the TFIIH-containing fractions derived from the 0.5 M wash of DEAE-Sephacel column (see Materials and Methods for details; 1× and 2× mean 5 and 10 μl of the activated GST-CDK_7 beads, respectively). Molecular weight markers (in thousands) and phosphorylated p53 are indicated on the left and right sides, respectively. The stars denote the possibly contaminating kinase(s) in TFIIH.

figure legends, were incubated with a 3′-end-labeled DNA fragment harboring one or two copies of the p53RE sequence (5,000 cpm, 0.1 to 1.0 ng of DNA/assay) for 30 min at room temperature. The reaction mixture contained 10 mM HEPES buffer (pH 7.5), 4 mM $MgCl_2$, 60 mM NaCl, 100 ng of poly(dI-dC), 0.1% NP-40, and 0.1 mM EDTA in a total volume of 20 μl. The complexes formed were separated by electrophoresis through a 5% native gel containing 4% glycerol and were detected by autoradiography.

RESULTS

p53 is phosphorylated by three different sources of TFIIH-associated kinases in vitro. To examine whether TFIIH phosphorylates p53, we purified p53 from baculovirus-infected Sf9 insect cells and the TFIIH proteins from HeLa nuclear extracts as described in Materials and Methods. Three different sources of TFIIH were used in the p53 phosphorylation assay: (i) TFIIH purified from HeLa nuclear extracts; (ii) TFIIH immunoprecipitated with anti-ERCC3 antibodies from the 0.5 M wash fractions of the phosphocellulose column; and (iii) GST-CDK_7 fusion protein activated with the 0.3 M nuclear extract fractions of the DEAE-Sepharose column. These three sources were separately incubated with highly purified p53 and [γ-^{32}P] ATP as substrates. As shown in Fig. 1, the phosphorylated products were analyzed by SDS-PAGE and autoradiography. All three preparations of TFIIH proteins specifically phosphorylated p53 in a dose-dependent manner (see lanes 2, 3, 5, 6, 8, and 9), as there was no detectable p53 phosphorylation in the absence of kinases (lane 1) or of p53 (lanes 4, 7, and 10). Also, the TFIIH-associated kinase proteins immunoprecipitated by both anti-ERCC3 and anti-cycH antibodies from H1299 cells and HeLa cells were able to phosphorylate p53 (data not shown). These results with three different preparations of TFIIH strongly suggest that p53 may be the substrate for the TFIIH-associated kinase activity.

p53 is phosphorylated by the CDK_7-cycH complex and stimulated in the presence of p36. Since the TFIIH-associated kinases used in the above experiment were only partially purified, it was possible that the p53 phosphorylation was due to some contaminating kinase activities. To rule out this possibility, we tested whether highly purified kinase components of TFIIH phosphorylate p53. To this end, the CDK_7-cycH-p36 trimeric and CDK_7-cycH dimeric complexes of TFIIH were purified to homogeneity after being produced in baculovirus-infected Sf9 cells. Figure 2A shows a silver-stained preparation (top panel) and Western blot analyses (two bottom panels) of these purified protein complexes.

The purified enzymes were used to measure kinase activity with four different substrates, p53, GST-CDK_2 (21), RNA polymerase II (47), and casein. The result is presented in Fig. 2B. In the absence of the kinases, there was no detectable phosphorylation in all these substrates alone (lanes 1 to 4). Consistent with the previous reports (20, 21, 38, 72), both the CDK_7-cycH dimer and CDK_7-cycH-p36 trimer complexes phosphorylated the GST-CDK_2 and RNA polymerase II substrates (lanes 7, 8, 12, and 13). However, there was no significant difference between the dimer and trimer kinase complexes in phosphorylating the two substrates (compare lanes 7 and 8 with lanes 12 and 13). In contrast, the phosphorylation of p53 by the dimer kinase was less efficient (lane 11) and was significantly stimulated in the presence of p36 (lane 6). This result not only demonstrates an important role for p36 in the efficient phosphorylation of p53 by the CDK_7-cycH complex but also suggests a possible p53-p36 interaction. That p53 phosphorylation was not due to contaminating CKs in the purified enzyme preparation was shown by the observation that casein was not phosphorylated by either the dimer or the trimer kinase complexes (lanes 9 and 14). It was also observed that when an increasing amount of the GST-CDK_2 protein was added to the reaction mixture, CDK_2 itself became more phosphorylated and simultaneously reduced the p53 phosphorylation by the CDK_7-cycH-p36 complex (data not shown), suggesting that these two substrates compete for the same enzyme.

Autophosphorylation of the CDK_7 component was seen when the highly purified dimeric protein was used (Fig. 2B, lanes 10 to 14). Interestingly, in the case of the trimeric complex, not only was the CDK_7 autophosphorylation elevated but also the phosphorylation of cycH and p36 was clearly visible (lanes 5 to 9). It is unlikely that these phosphorylations are catalyzed by other contaminating kinases for the following reasons: (i) the kinases are of high purity (Fig. 2A), and (ii) the substrates (GST-CDK_2 and p53) and the CDK_7-cycH-p36 polypeptides compete for the same kinase activity (Fig. 2B, compare lane 5 with lanes 6 and 7). However, the autophosphorylation was not apparent when partially purified TFIIH was used in a kinase assay (Fig. 1). Thus, whether the autophosphorylation of the CDK_7-cycH-p36 trimeric complex is important for auto-activation of this kinase in vivo remains an open question.

The inactive K41A mutant of CDK_7 complexed with cycH and p36 is also defective in phosphorylation of p53. To further confirm that the phosphorylation of p53 was truly due to the kinase activity from CDK_7, not from a contaminated kinase in the purified proteins, a CDK_7 mutant was used. This mutant contains a mutation from Lys41 to Ala (K41A) at the ATP-binding site and was previously shown to be inactive in phosphorylation of CDK_2 (21). The baculovirus harboring K41A was used to cotransfect Sf9 cells with the viruses containing

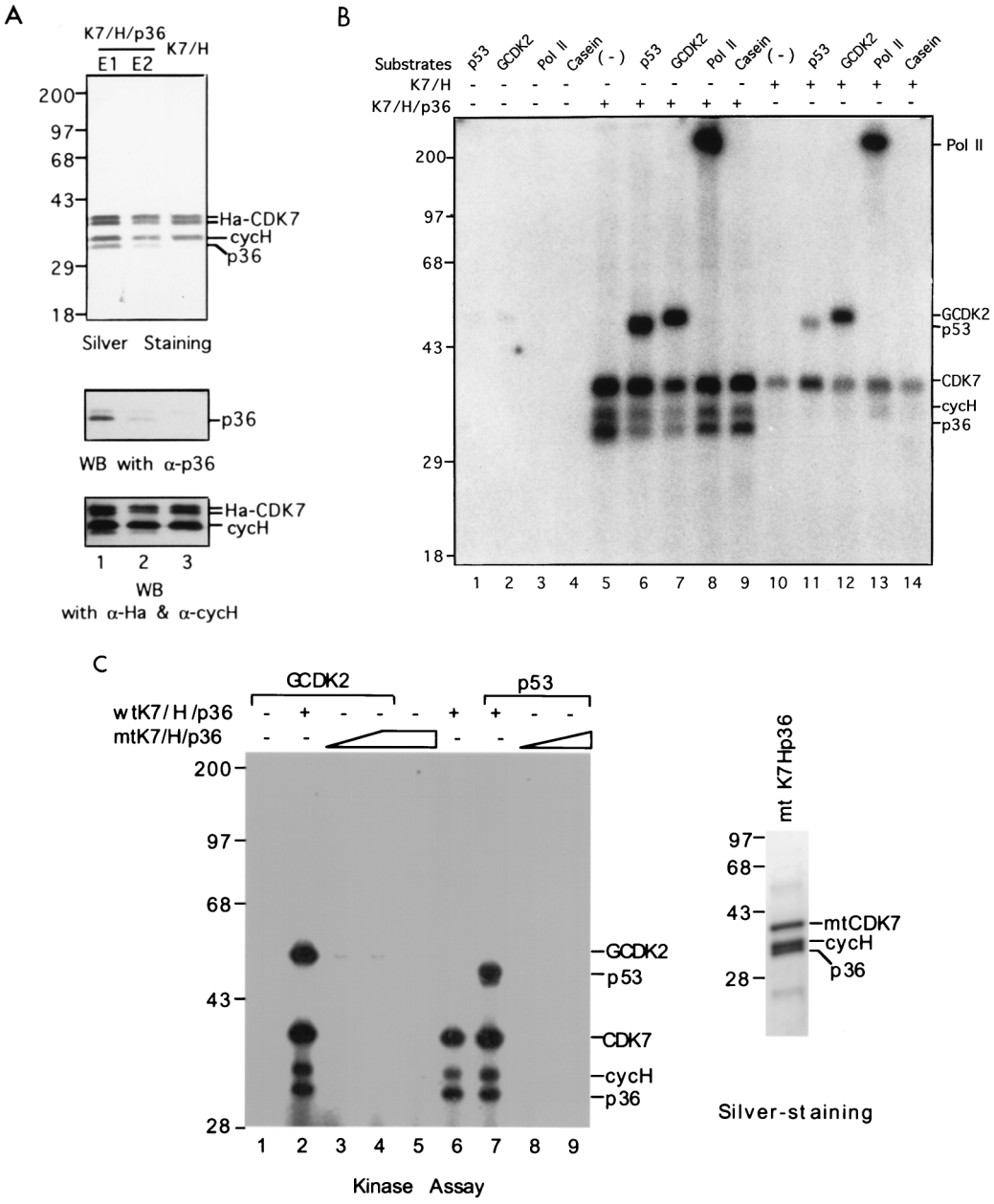

FIG. 2. p53 is phosphorylated by the CDK_7-cycH-p36 trimeric complex. (A) Purification of the CDK_7-cycH-p36 trimeric and CDK_7-cycH dimeric complexes. The protein complexes were purified on immunoaffinity (12CA5) columns and analyzed by silver staining (top) and Western blotting (middle and bottom) as described in Materials and Methods. As indicated at the top, lanes 1 and 2 are the two elutions of the trimeric complex from the 12CA5 column and lane 3 is the elution of the dimeric complex from a separate 12CA5 column. A 5-μl volume of the eluted proteins was loaded onto an SDS–10% polyacrylamide gel. α-p36 indicates anti-p36 monoclonal antibodies; α-Ha indicates anti-HA peptide 12CA5 antibodies; and α-cycH indicates anti-cycH polyclonal antibodies. (B) The p53 protein is efficiently phosphorylated by the CDK_7-cycH-p36 trimeric complex but not by the CDK_7-cycH dimeric complex. The substrates and kinases used in the experiment are indicated at the top. We used 40 ng of p53, 100 ng of $GCDK_2$ (GST-CDK_2 fusion protein), 80 ng of Pol II (RNA polymerase II), and 100 ng of casein in the kinase assay; 10 ng of K7/H (the CDK_7-cycH dimer) and K7/H/p36 (the CDK_7-cycH-p36 trimer) were used in the assay. The molecular weight markers (in thousands) and phosphorylated protein products are shown on the left and right sides, respectively. (C) The K41A mutant of CDK_7 does not phosphorylate CDK_2 and p53 in vitro. The purified trimeric complex (5 μl loaded) containing the K41A mutant of CDK_7, cycH, and p36 analyzed on a silver-stained SDS–9% polyacrylamide gel is shown on the right. The phosphorylation reaction with GCDK2 and p53 as substrates is shown on the left. The amounts of the kinase complexes and substrates used in the experiment were the same as those used in panel B, except that the mutant CDK_7 complex was titrated from 10 to 20 ng (lanes 3, 4, 8, and 9).

cycH and p36. The trimeric K41A-cycH-p36 complex was purified by the aforementioned method and analyzed on a silver-stained SDS gel (Fig. 2C, right panel). A kinase reaction on GCDK2 and p53 was performed with the K41A complex and the wild-type CDK_7 complex, as shown in the left panel of Fig. 2C. Consistent with the previously reported results (21), the mutant K41A complex was not active in phosphorylation of GCDK2 (lanes 3 and 4). Similarly, phosphorylation of p53 was hardly detectable in the presence of the K41A complex (lanes 8 and 9), suggesting that CDK_7 is the catalytic component of the kinase complex responsible for p53 phosphorylation. In contrast, the wild-type CDK_7 efficiently phosphorylated both

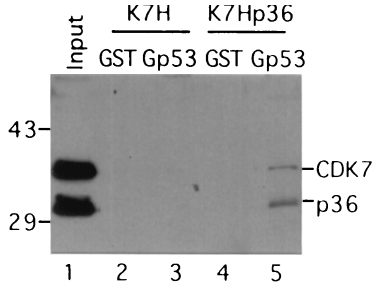

FIG. 3. p53 interacts with the CDK_7-cycH-p36 trimeric complex but not with the CDK_7-cycH dimeric complex. The GST-protein-protein interaction assay was done as described in Materials and Methods. The proteins employed in this assay are indicated at the top. Approximately 100 ng of the kinase complexes as well as 300 ng of GST-p53 and GST proteins were used.

GCDK2 and p53 (lanes 2 and 7). Noticeably, autophosphorylation of the trimeric mutant complex was also reduced drastically (lanes 3, 4, 8, and 9), compared with the wild-type proteins (lanes 2, 6, and 7). Accordingly, the doublet of CDK_7 that can be seen in Fig. 2A disappeared in the purified K41A-cycH-p36 complex (the right panel of Fig. 2C), probably due to the absence of autophosphorylation of the mutant CDK_7. Taken together, these results demonstrate that the phosphorylation of p53 is indeed catalyzed by the CDK_7 component of the trimeric complex. The K41A mutant was defective in phosphorylation of both p53 and GCDK2.

p53 interacts with the CDK_7-cycH-p36 trimeric complex but not the CDK_7-cycH dimeric complex. The above study suggests that p36 may directly interact with p53. To test the possibility, a GST fusion protein-protein interaction assay was carried out with the GST-p53 fusion protein, which contains a human wt p53 of full length (46). The GST-p53 beads were incubated with the purified CDK_7-cycH dimer and CDK_7-cycH-p36 trimer complexes at room temperature for 1 h and washed as described in Materials and Methods. The bound proteins were detected by Western blotting with anti-p36 and anti-CDK_7 antibodies. As shown in Fig. 3, p53 bound to the trimer kinase complex but not to the dimer complex (compare lanes 3 and 5), indicating that p36 is crucial for the CDK_7-cycH-p36 interaction with p53. This interaction was specific, since neither the dimer nor the trimer complex bound to the GST protein (lane 4). In addition, purified mixtures of p36 alone and p53 bound to each other in vitro and could be coimmunoprecipitated (results not shown). Taken together, these results suggest that the direct p36-p53 interaction may account for the efficient phosphorylation of p53 by the CDK_7-cycH-p36 complex in vitro.

The C-terminal domain (aa 311 to 393) of p53 is phosphorylated by the ternary complex of CDK_7-cycH-p36. In an attempt to map the location of p53 which is phosphorylated by the CDK_7-cycH-p36 complex, we used short peptide fragments of p53 in the kinase assay. A set of the substrate competition experiments was conducted to examine the effect of these p53 peptides on the p53 phosphorylation by this kinase. As shown in Fig. 4, the C-terminal aa 311 to 393 of p53 reduced the p53 phosphorylation by the ternary kinase complex in a dose-dependent manner (Fig. 4A, lanes 1 to 4, and Fig. 4B, lanes 2 to 5), and the peptide itself became phosphorylated by the kinase (Fig. 4B, lanes 3 and 4). In contrast, the N-terminal peptides (aa 10 to 25 and 60 to 92) of p53 had no significant effect on the p53 phosphorylation, even though a 1,000-fold excess (in molar ratio) of the peptides was used (Fig. 4A, lanes 5 and 6). In the absence of full-length p53, the 311 to 393 p53 peptide was highly phosphorylated by the kinase (Fig. 4B, lane 5) whereas

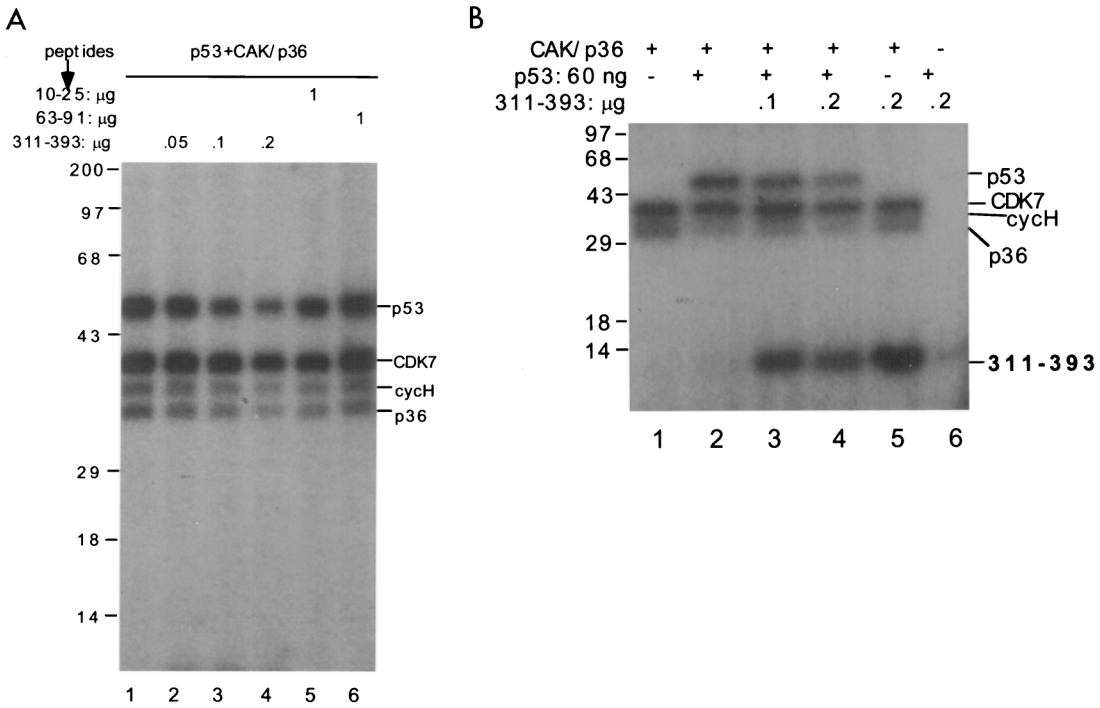

FIG. 4. The C-terminal domain of p53 is phosphorylated by the CDK_7-cycH-p36 complex. The kinase and the amount of peptides are indicated at the top of the two panels. The kinase/peptide competition assay was conducted as described in Materials and Methods. (A) We used 60 ng of p53 and 10 ng of the CAK/p36 kinase (the CDK_7-cycH-p36 complex). Phosphorylated proteins were subjected to electrophoresis on an SDS–10% polyacrylamide gel (large gel). (B) A similar kinase/peptide competition assay was done, except that only the aa 311 to 393 peptide was used and the proteins were separated on a mini-SDS–10% polyacrylamide gel to visualize the phosphorylated peptides.

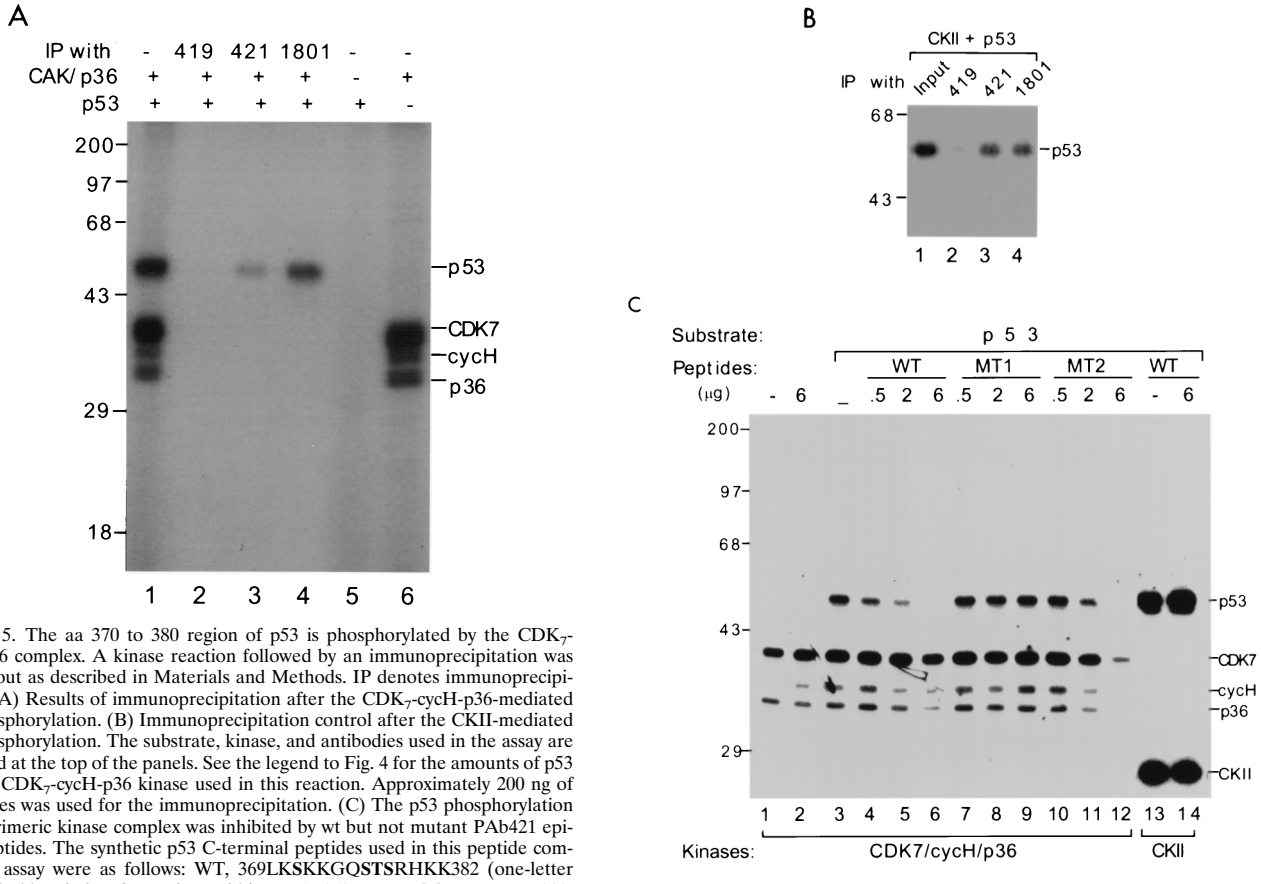

FIG. 5. The aa 370 to 380 region of p53 is phosphorylated by the CDK_7-cycH-p36 complex. A kinase reaction followed by an immunoprecipitation was carried out as described in Materials and Methods. IP denotes immunoprecipitation. (A) Results of immunoprecipitation after the CDK_7-cycH-p36-mediated p53 phosphorylation. (B) Immunoprecipitation control after the CKII-mediated p53 phosphorylation. The substrate, kinase, and antibodies used in the assay are indicated at the top of the panels. See the legend to Fig. 4 for the amounts of p53 and the CDK_7-cycH-p36 kinase used in this reaction. Approximately 200 ng of antibodies was used for the immunoprecipitation. (C) The p53 phosphorylation by the trimeric kinase complex was inhibited by wt but not mutant PAb421 epitope peptides. The synthetic p53 C-terminal peptides used in this peptide competition assay were as follows: WT, 369LK**S**KKGQ**ST**SRHKK382 (one-letter standard abbreviation for amino acids); MT1, 369LK**A**KKGQ**AT**ARHKK382; MT2, 365H**AA**HLKSKKGQS**A**SR379. The amounts of the peptides used are indicated at the top of the panel. CKII was used as a control. The amounts of p53 and the kinase were the same as those in Fig. 4.

phosphorylation was not detectable in the absence of the kinase (lane 6). Notably, the higher concentration of peptides required for complete inhibition of p53 phosphorylation by the trimeric kinase complex (see Fig. 5C) suggests that the wt p53 protein may be a better substrate than the short peptide. Together, these results clearly demonstrate that the C-terminal domain (aa 311 to 393) of p53 is the primary target for the CDK_7-cycH-p36 complex.

Serines 371, 376, 378, and 392 are the potential target sites for the CDK_7 kinase in vitro. To further define the phosphorylation sites of p53 by this kinase, the effect of anti-p53 monoclonal antibodies on the p53 phosphorylation was exploited by using PAb421 (its epitope is at the aa 370 to 378 region) and PAb1801 (epitope at the N-terminal domain). PAb421 dramatically inhibited the p53 phosphorylation by CDK_7-cycH-p36, while PAb1801 had no effect (data not shown), suggesting that the 421 epitope region may encompass the kinase binding or phosphorylation site. The large antibody molecules might sterically inhibit phosphorylation by the kinase at sites adjacent to or further away from the antibody epitope. To avoid this complication, we took advantage of the observation that PAb421 fails to bind to its epitope site (aa 370 to 378) when one to three serines in this epitope sequence are phosphorylated, while PAb1801 is not blocked by phosphorylation of any kinase (30). A phosphorylation reaction of p53 was carried out with either CDK_7-cycH-p36 or CKII, followed by immunoprecipitation with PAb1801 and PAb421. The result of this experiment is presented in Fig. 5. Most of the phosphorylated p53 molecules were immunoprecipitated by PAb1801 but not by PAb421 (Fig. 5A, compare lane 1 with lanes 4 and 3). By contrast, PAb1801 and PAb421 immunoprecipitated equal amounts of the CKII-phosphorylated p53 protein (Fig. 5B), consistent with the previous report (30). This result is most consistent with the possibility that phosphorylation occurs at or around the region (aa 370 to 378) of the PAb421 epitope, preventing the epitope-antibody interaction. This experiment also eliminates the interpretation that the aa 370 to 378 site is used for binding of the kinase. As a control, none of the phosphorylated p53 molecules were precipitated by PAb419 (lane 2), which is specifically directed against the Tag protein of simian virus 40 (28). In addition, the CDK_7-cycH-p36 kinase did not phosphorylate the alternatively spliced form of murine p53 (P53AS) (46a), which lacks the C-terminal 26 aa and thus the PAb421 epitope (4). Based on these studies, it appears clear that the aa 370 to 378 region of p53 is the target site for the CDK_7-cycH-p36 kinase.

To precisely determine the phosphorylation sites, a second peptide competition assay was performed with three different p53 C-terminal peptides. As indicated in the legend of Fig. 5C, these synthetic p53 peptides of 14 residues are wild type from aa 369 to 382; mutant 1, which contains the three mutated sites S371A, S376A, and S378A; and mutant 2, which contains the three mutated sites S366A, S367A, and T377A. These peptides were preincubated with the CDK_7-cycH-p36 complex for 15 min at 4°C before the addition of p53 and ATP substrates. As shown in Fig. 5C, the wild-type p53 peptide inhibited the phosphorylation of p53 by the trimeric kinase complexes in a dose-

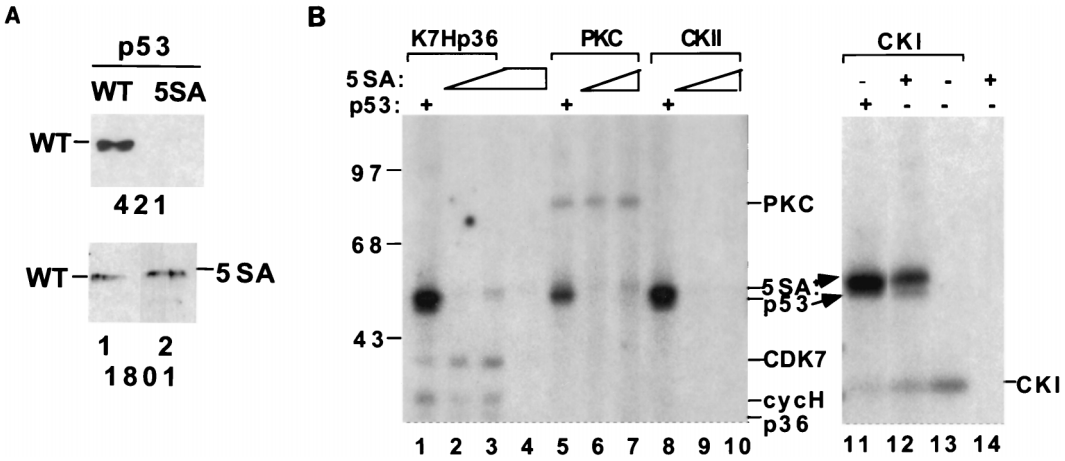

FIG. 6. The 5SA mutant of p53 is not phosphorylated by the CDK_7-cycH-p36 kinase. (A) Western blot analysis of the wt p53 and mutant 5SA proteins purified from the baculovirus expression system (see Materials and Methods for details). 5SA is a p53 mutant with replacement of Ser371, Ser376, Ser378, Ser392, and Thr377 by five alanines. The top panel indicates the immunoblotting with PAb421, and the bottom panel denotes the immunoblotting with PAb1801. For both p53 and 5SA, 60 ng was loaded onto an SDS–10% polyacrylamide gel. (B) Results from in vitro kinase assays with p53 (60 ng) and 5SA (30 and 60 ng) as substrates. We used 20 ng of the CDK_7-cycH-p36 kinase (K7Hp36), 40 μU of PKC, 0.1 mU of CKII, and 0.9 U of CKI in the reactions, as indicated at the top. Molecular weight markers (in thousands) are indicated on the left, and phosphorylated proteins are indicated on the right.

dependent fashion (lanes 3 to 6), as did mutant 2 (lanes 10 to 12). When 6 μg (100-fold more than the amount of p53) of these two peptides was used, the p53 phosphorylation was hardly detectable (lanes 6 and 12). This inhibition was specific, since the phosphorylation of p53 by CKII was not affected by the wild-type p53 peptide, although 6 μg of this peptide was used (lanes 13 and 14). In striking contrast, the mutant 2 peptide had no effect on the p53 phosphorylation by the CDK_7 kinase complexes (lanes 7 to 9), suggesting that serine residues 371, 376, and 378 are the potential targets for this kinase. Consistent with these data in Fig. 5A, these results suggest that the CDK_7-cycH-p36 kinase may target serines 371, 376, and 378 as potential phosphorylation sites of p53.

Next, a p53 mutant was generated with five potential phosphorylation sites replaced with alanines at serines 371, 376, 378, and 392 as well as threonine 377. This mutant is referred to as 5SA. As shown in Fig. 6A, the 5SA protein purified from a baculovirus expression system, unlike wt p53 (lane 1), immunoreacted with only PAb1801 (bottom panel, lane 2) but not PAb421 (top panel) antibodies in a Western blot analysis, demonstrating the PAb421 specificity for these altered residues. The same amounts of wt p53 and 5SA proteins (bottom panel) were used in a kinase assay comparing four different kinases (Fig. 6B). Like PKC (lanes 6 and 7) and CKII (lanes 9 and 10), the CDK_7-cycH-p36 trimeric kinase complex was unable to phosphorylate the 5SA protein to a significant level (lanes 2 and 3) compared with wt p53 as a substrate (lanes 1, 5, and 8). This result is consistent with the previous reports showing that PKC targeted serines 371, 376, and 378 (3) while CKII phosphorylated serine 392 (30). These results also indicate that the serines 371, 376, 378, and 392 and perhaps threonine 377 may be the target sites for the CDK_7 kinase of TFIIH. Based on the result in Fig. 5C, Thr377 is unlikely to be a CDK_7 site because the MT2 peptide with a mutation of T377A acted like the wt p53 peptide in the kinase/peptide competition assays. Thus, the four serines 371, 376, 378, and 392 in the C terminus of p53 are most probably the potential sites for the CDK_7 kinase of TFIIH. The idea that these serines are binding targets for the CDK_7 kinase which phosphorylates elsewhere in the molecule is ruled out by the failure of PAb421 to bind to the CDK_7-phosphorylated p53 product.

The finding that the CDK_7-cycH-p36 kinase complex phosphorylates the PAb421 epitope and Ser371, Ser376, and Ser378 raised the question whether this purified kinase, although very pure, was contaminated by a trace amount of PKC, which was previously reported to phosphorylate the same region of p53 (at Ser371, Ser376, and Ser378) (3). To exclude this possibility, a PKC inhibitor (a pseudosubstrate) was used in the p53 phosphorylation assay. As shown in Fig. 7, this inhibitor specifically inhibited both the autophosphorylation and p53 phosphorylation by PKC in a dose-dependent manner (lanes 6 to 8). In contrast, its effect on the p53 phosphorylation and autophosphorylation by the CDK_7-cycH-p36 kinase complex was not significant (lanes 3 to 5), since there was only a slight reduction (less than twofold) of CDK_7-mediated p53 phosphorylation by the same high dose (600 ng) of PKC inhibitor that completely eliminated the activity of PKC (compare lane 5 with lane 8). Notably, there was no detectable autophosphorylation of PKC in the CDK_7 kinase reactions (Fig. 7, lanes 1 and 3 to 5; Fig. 2, 4, and 5), even after longer exposure (data not shown). Hence, the p53 phosphorylation is indeed catalyzed by the CDK_7 protein kinase in the ternary complex.

The phosphorylation of p53 by the ternary complex of CDK_7-cycH-p36 enhances the ability of p53 to bind to DNA specifically. Having found that p53 can be phosphorylated by the CDK_7-cycH-p36 kinase complex, we examined the effect of p53 phosphorylation by the kinase on p53's sequence-specific DNA-binding activity by using an electrophoretic gel mobility shift assay. In the presence or absence of ATP, p53 was preincubated at 30°C for 20 min with PKC or the CDK_7-cycH-p36 kinase, respectively. These mixtures were added to the reaction cocktails containing DNA probes with two copies of the p53-binding element derived from the $p21^{waf1}$ promoter (15). DNA-p53 complexes were subjected to electrophoresis on a native gel. The result is presented in Fig. 8A. Neither CDK_7-cycH-p36 (lane 2) nor PKC (lane 6) bound to the DNA probes. However, in a dose-dependent manner, both the CDK_7-cycH-p36 kinase and PKC stimulated the DNA-binding activity of p53 dramatically, although to different extents (lanes 4 and 5

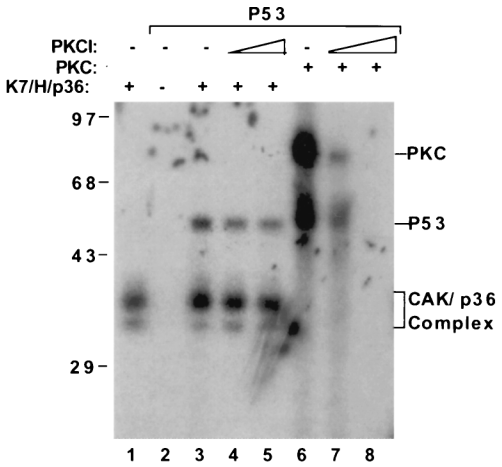

FIG. 7. Phosphorylation of p53 by the CDK_7-cycH-p36 complex is not due to contaminating PKC protein. The substrates, kinases, and a PKC inhibitor used in this reaction are indicated at the top. We used 80 μU of PKC and 300 and 600 ng of the PKC inhibitor. The same amounts of p53 and CDK_7-cycH-p36 complex as in Fig. 4 were used in the reaction.

and lanes 7 and 8). This difference between CDK_7 and PKC in the stimulation of p53 DNA-binding activity may be due to (i) a difference in the stoichiometry of p53 phosphorylation by these two enzymes or (ii) different phosphorylation sites of p53 targeted by them, even with some common sites. A careful analysis must be done to address these issues. Nevertheless, the stimulation was due to the phosphorylation of p53 by these kinases, since p53 bound weakly to the specific DNA probes without ATP even in the presence of the CDK_7-cycH-p36 kinase (lane 5) and PKC (lane 9). These DNA-protein complexes are specific for p53 because (i) anti-p53 antibodies supershifted the complexes and (ii) the complexes were specifically competed by nonlabeled p53RE-containing DNA fragments but not by a mutated p53RE fragment which was not bound by p53 (data not shown). To rule out the possibility that the activation of the DNA-binding activity of p53 by the CDK_7-cycH-p36 kinase was caused by a trace amount of contaminating PKC, the PKC inhibitor was included in the following DNA-binding reaction mixture. As shown in Fig. 8B, the PKC inhibitor strongly and specifically reduced the stimulatory effect of PKC on the formation of the p53-DNA complexes (compare lane 6 with lanes 4 and 5). Consistent with previous experiments (Fig. 7), this inhibitor did not show any effect on the activation of the DNA-binding activity of p53 by the CDK_7-cycH-p36 kinase (compare lane 9 with lanes 7 and 8). As a control, the PAb421 epitope peptide, when added to the reaction, also enhanced the DNA-binding of p53 greatly (lane 2), in agreement with the previous reports (32). The heterogeneous p53-DNA complexes (Fig. 8) are most probably due to the two copies of p53-binding sites in the DNA probes used in these experiments. Taken together, these results demonstrate that the phosphorylation of p53 by the CDK_7-cycH-p36 kinase ac-

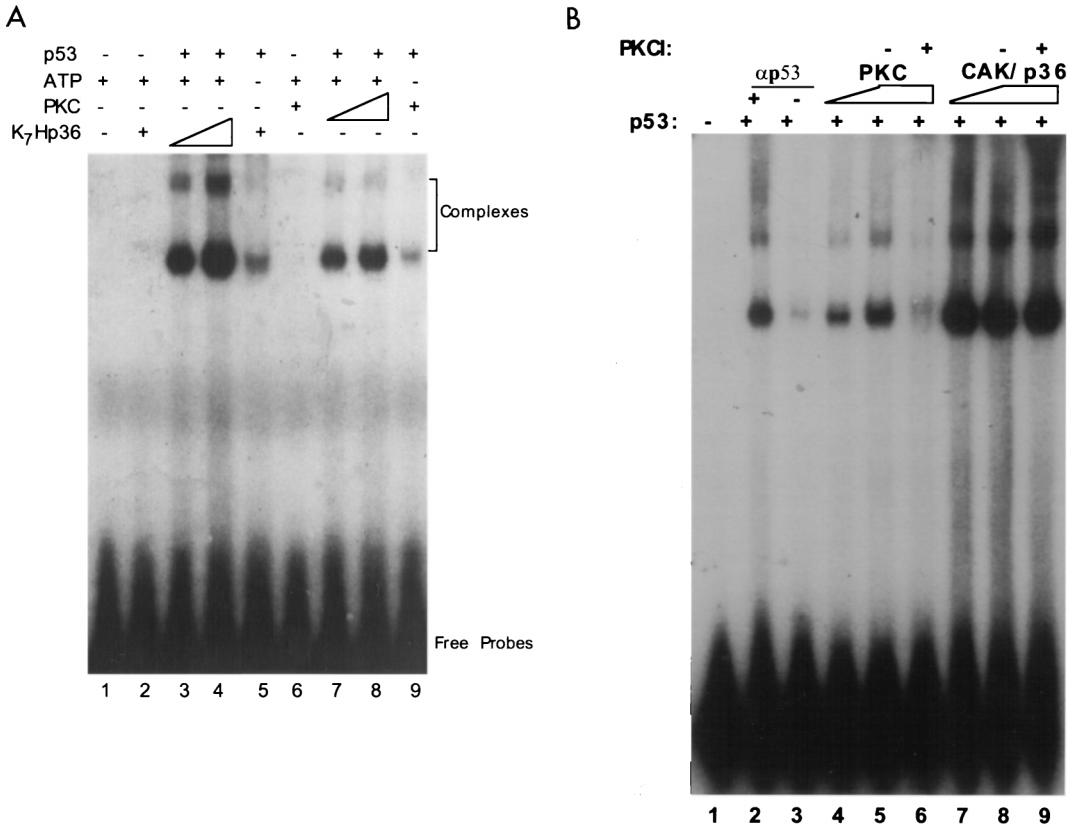

FIG. 8. The p53 phosphorylation by the CDK_7-cycH-p36 complex stimulates the sequence-specific DNA-binding activity of p53. (A) p53 was preincubated, in the presence or absence of 1 mM ATP, with 40 and 80 μU of PKC or 10 and 30 ng of the CDK_7-cycH-p36 complex at 30°C for 30 min. The mixture was added to a DNA-binding cocktail containing ^{32}P-labeled DNA probes with two copies of p53 derived from the $p21^{waf1}$ promoter. DNA-protein complexes were subjected to electrophoresis on a native gel. The reagents used in the reactions are shown at the top. (B) Experiment similar to that in panel A, except that 300 ng of PKC inhibitor (PKCI) was used as indicated at the top. The PAb421 epitope (100 ng) was used here as a positive control (lane 2).

tivates the ability of p53 to specifically bind to its DNA-responsive elements. This also suggests a possible role for the TFIIH-associated kinase in regulating the functions of p53 in response to cellular stress.

DISCUSSION

This paper demonstrates that p53 is phosphorylated by the TFIIH-associated kinase in vitro, and this conclusion is supported by several lines of evidence. First, p53 is phosphorylated in vitro by different sources of TFIIH (Fig. 1). Also, by using highly purified kinase components of TFIIH, the phosphorylation of p53 is most efficient in the presence of the CDK_7-cycH-p36 trimer complex (Fig. 2B). The stimulation of p53 phosphorylation in the presence of p36 may be explained by the binding between p53 and p36 (Fig. 3 and unpublished data). Moreover, the inactive CDK_7 mutant K41A (21), when complexed with cycH and p36, did not phosphorylate p53 at all (Fig. 2C), strongly supporting a catalytic role for the CDK_7 subunit in this phosphorylation. Finally, contamination of the CDK_7 kinase preparation by CKII and PKC kinases was unlikely, since the purified CDK_7 kinase did not phosphorylate the CKII substrate casein (Fig. 2B) and a PKC inhibitor did not significantly affect p53 phosphorylation (Fig. 7 and 8B). Therefore, it can be concluded that the kinase components, CDK_7-cycH-p36, of TFIIH phosphorylate p53.

Our study further shows that the C-terminal domain (aa 311 to 393) of p53 is a primary target for the TFIIH-associated kinase activity. This stems from the following avenues of data. (i) The phosphorylation of p53 by the CDK_7-cycH-p36 kinase was apparently competed in a dose-dependent fashion by its C-terminal polypeptide but not by two N-terminal peptides (Fig. 4A). (ii) The C-terminal domain of p53 itself was phosphorylated by the CDK_7-cycH-p36 kinase in vitro (Fig. 4). (iii) p53 phosphorylation by the CDK_7 kinase blocked the immunoreactivity of p53 with the p53-specific PAb421, whose epitope resides in the C-terminal region between aa 370 and 380 (30), but not with the anti-p53 N-terminal PAb1801 (Fig. 5A). (iv) The CDK_7-mediated p53 phosphorylation was consistently inhibited by a wt PAb421 epitope peptide as well as a mutant peptide containing the three point mutations S366A, S367A, and T377A but not by a mutant peptide containing the three mutations S371A, S376A, and S378 (Fig. 5C). Taken together, these results suggest that the primary phosphorylation targets are within or proximal to the PAb421 epitope region, a region found phosphorylated in vivo (30, 51). In addition, the serines 371, 376, 378, and 392 may be the potential targets, since a mutant with replacement of these serines by alanines was not phosphorylated by the CDK_7 kinase of TFIIH (Fig. 6). Phosphorylation modification of p53 at its C-terminal domain has been shown to positively regulate its sequence-specific DNA-binding activity (3, 30, 52, 75). In agreement with that observation, the CDK_7-mediated phosphorylation of p53 also dramatically enhanced the ability of this protein to bind to its DNA response element (Fig. 8). Hence, this study suggests that the CDK7-cycH-p36 trimeric kinase phosphorylates the p53 protein at its C-terminal domain and that this in turn activates p53 for sequence-specific DNA binding.

Previous studies have shown that p53 interacted with three different components of TFIIH, ERCC2, ERCC3, and p62, in vitro (73, 77), and this inhibited the helicase activity of TFIIH. p53 mutants arising in cancer cells no longer alter the TFIIH helicase activity (73). In addition, the two helicases, ERCC2 and ERCC3, of TFIIH were found to be essential for p53-induced apoptosis (74). The finding reported here that p53 can be phosphorylated by the CDK_7-cycH-p36 trimeric complex of TFIIH adds to this scenario another functional link between p53 and TFIIH. Interestingly, p53 levels were reported to increase after UV irradiation of cells when DNA lesions occurred in actively transcribed genes rather than the entire genome (80). Also, p53 induction by UV irradiation correlated with a significant reduction of total mRNA synthesis in cells, indicating that blockage of RNA polymerase II function may serve as a signal for UV-induced p53 activation (44). Collectively, then, these data lead to a testable model for the cooperation of TFIIH with p53 during cellular responses to DNA damage (Fig. 9). Since actively transcribed genes in the cell are subject to rapid repair, RNA polymerase II, when meeting the lesion site, could well pause at this site. As an essential component for NER (1), TFIIH might also bind to the stalled polymerase-DNA complex (13). p53 could then join the polymerase-TFIIH complex, perhaps by contacting with TFIIH and/or by binding to the damaged DNA (39). Subsequent phosphorylation of p53 by TFIIH in this complex could then release and activate p53 for transcription at p53-responsive elements. In some cell types, such as those NER-impaired XPA and CSB cells (44), the activated p53 protein induces apoptosis, probably by upregulating BAX-1 (53) or IGF-BP3 (7) genes. In other cells, p53 activation results in cell growth arrest, in part by transcriptionally activating $p21^{waf/cip1}$ (15) and possibly facilitating DNA repair to occur prior to DNA replication, protecting genomic integrity. Intriguingly, an oxidation inhibitor, *N*-acetylcysteine, was reported to specifically attenuate the cellular p53 response to UV but not γ irradiation by reducing the number of cells with high p53 level (63), im-

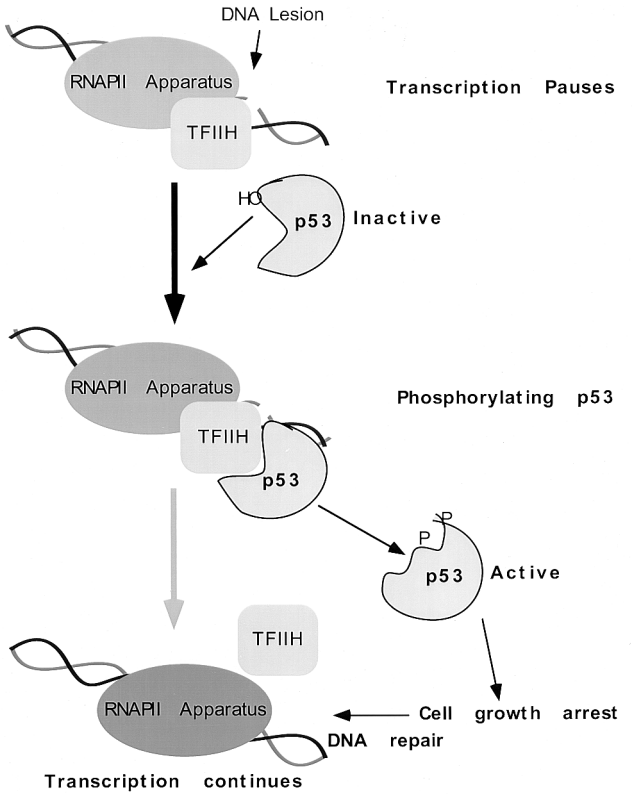

FIG. 9. Hypothetical model for how p53 may be phosphorylated by the TFIIH-associated kinase in response to DNA damage.

plicating oxidative stress as one signal in the UV-responsive p53 pathway. It seems reasonable that both oxidative stress and UV repair intermediates signal to p53 when DNA damage has occurred and that the p53 levels respond accordingly.

Many kinases which can phosphorylate p53 in vitro have been identified (3, 5, 18, 30, 33, 49, 50, 52, 75). One possibility is that p53 is regulated by multiple kinases in vivo, each acting at a specific phase of the cell cycle or under various situations of cellular stress. It is conceivable that these stress signals may use alternate pathways to activate p53. For instance, ATM (ataxia-telangiectasia-mutated) may be an important upstream component of the p53 signaling pathway in response to γ irradiation (36). Recently, p53 was shown not to respond to γ irradiation in ATM-deficient cells, whereas it was inducible at the protein level by UV-mediated DNA damage (2, 79). This observation clearly distinguishes two different mechanisms for UV- and γ-induced p53 accumulation. Obviously, it is crucial to unravel whether ATM can phosphorylate p53 in cells and why this phosphorylation responds to γ but not UV irradiation.

Elucidating the role of many potential p53 regulatory kinases is essential for understanding the mechanisms by which p53 is regulated. Unfortunately, some of these kinase activities that have been demonstrated in vitro may not necessarily lead to relevant in vivo function of the kinases in the p53 pathway. For instance, PKC was previously found to phosphorylate p53 at its PAb421 epitope region and to stimulate its specific DNA-binding activity in vitro (3). A recent report nevertheless showed that PKC did not phosphorylate p53 in vivo and that a PKC activator, 12-*O*-tetradecanoylphorbol-13-acetate, did not stimulate the p53 phosphorylation by PKC either (51), suggesting that PKC may not be responsible for p53 phosphorylation in cells. However, although the murine p53 serines 370 to 372 (corresponding to human Ser376, Thr377, and Ser378) were not phosphorylated by PKC in vivo (51), experiments in vitro demonstrated the positive regulatory effect of phosphorylation of these residues on p53. DNAPK was also shown to phosphorylate the N-terminal serines 15 and 37 of p53 (18). However, recent studies have shown that in the presence of an inactive DNAPK mutant, p53 is fully functional for both G_1 arrest and apoptosis in response to radiation (6, 26, 27, 56, 61). As a result, the role played by DNAPK in p53-mediated response to radiation remains ambiguous. Either these kinases play no role in vivo or there is a considerable redundancy of p53 modification in vivo. The exact interpretations here remain ambiguous.

Phosphorylation of p53 may represent an indispensable component of the UV-induced DNA damage pathway that modulates the multiple functions of p53. Other pathways for p53 activation, such as binding to damaged or single-stranded DNA (34, 39), to other proteins (31), or via redox modulation (31), may also be used for different types of DNA damage, in various cell types, and at different stages of the cell cycle. Clearly, successful elucidation of the regulatory role of the kinases, including TFIIH, in modulating the physiological functions of p53 will require additional systematic and intensive investigations and will prove essential for understanding the processes of cell growth regulation in vivo.

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