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Phosphorylation sites in the amino-terminal region of mouse p53

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ABSTRACT **Phosphorylation is an attractive mechanism for regulating the functions of p53. The $p34^{cdc2}$ kinase, which is involved in regulation of the cell cycle, phosphorylates serine-315 of human p53 *in vitro*. Casein kinase II phosphorylates serine-389 of mouse p53 *in vitro*. The amino-terminal region of mouse p53 contains a cluster of potential serine phosphorylation sites. Those sites have been proposed to be sites for phosphorylation by a double-stranded DNA-dependent kinase (DNA-PK) from HeLa cells and can be dephosphorylated by protein phosphatase 2A. To identify *in vivo* phosphorylation sites in the amino-terminal region of mouse p53, we mutated potential phosphorylation sites and analyzed the mutant proteins by tryptic phosphopeptide mapping. We identified serine-7, -9, -18, and -37 as *in vivo* phosphorylation sites. We further showed that mouse p53 expressed in bacteria is phosphorylated by DNA-PK on amino-terminal serine residues *in vitro*.**

The p53 protein can associate with transforming proteins of several DNA tumor viruses, including the simian virus 40 (SV40) large tumor (T) antigen, the E1b protein of adenovirus 5, and the E6 proteins of human papilloma virus types 16 and 18 (1). This association may alter or abolish the normal function of p53. Mutant forms of p53 are found in many human tumors (2).

Wild-type and mutant p53 proteins differ in several respects. Wild-type p53 behaves as a growth suppressor, whereas mutant p53 can immortalize primary cells and cooperate with activated *ras* to transform primary rat embryo fibroblasts (3, 4). Wild-type p53 inhibits SV40 origin-dependent DNA replication *in vitro* and *in vivo*, whereas some mutant forms of p53 do not (5, 6). Wild-type p53, linked to the DNA binding domain of the yeast Gal4 protein, displays transactivating activity in both yeast and mammalian cells (7, 8). The effects of p53 on DNA replication and transcription suggest that p53 may affect cell growth by inhibiting DNA synthesis or by regulating the expression of cellular genes.

Phosphorylation is an attractive mechanism for the regulation of p53 functions. There are two major serine phosphorylation sites in the carboxyl-terminal region of the protein (serine-312 and -389 in mouse p53) and a cluster of potential serine phosphorylation sites in the amino-terminal region (9–11). The $p34^{cdc2}$ kinase, which is involved in regulation of the cell cycle, phosphorylates serine-315 of human p53 (equivalent to serine-312 of mouse p53) *in vitro* (12). Casein kinase II, which is stimulated in response to mitogens and phosphorylates several nuclear oncoproteins, phosphorylates serine-389 of mouse p53 *in vitro* (13). Serine residues in the amino-terminal region of p53 have been suggested as potential targets for phosphorylation by a double-stranded DNA-dependent kinase (DNA-PK) from HeLa cells (14, 15) and for dephosphorylation by protein phosphatase 2A (16).

To further understand the possible regulation of p53 by phosphorylation, we set out to identify *in vivo* phosphorylation sites in the amino-terminal region of mouse p53. We mutated potential phosphorylation sites and analyzed the mutant proteins by tryptic phosphopeptide mapping. We identified serine-7, -9, -18, and -37 as the *in vivo* phosphorylation sites in the amino-terminal region. We further showed that DNA-PK phosphorylates p53 expressed in bacteria on amino-terminal serines *in vitro*.

MATERIALS AND METHODS

Cell Lines and Plasmid Constructs. COS cells, monkey cells expressing SV40 T antigen, were used to express wild-type and mutant mouse p53 proteins. The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum. Plasmids pCMV/Nc9 and pCMV/c5 encode wild-type and mutant p53, respectively, under the control of the cytomegalovirus (CMV) immediate-early promoter (3). p53 retroviral constructs, pLNL/XHCp53wt and pLNL/XHCp53mt, were constructed by cloning the *Hin*dIII/*Eco*RI fragment of pCMV/Nc9 or pCMV/c5, which encodes the entire p53 cDNA under the control of the CMV promoter, into the retroviral vector pLNL (17). pLNL contains neomycin phosphotransferase and β-lactamase genes for drug selections.

Mutation of Potential Phosphorylation Sites. The 3.5-kilobase *Hin*dIII/*Kpn* I fragment, encoding intron I and the amino-terminal half of p53 cDNA, was transferred into M13mp19, and the single-stranded phage DNA containing the coding strand of p53 cDNA was purified. Mutation of potential serine phosphorylation sites to alanine residues was carried out by using an oligonucleotide-directed *in vitro* mutagenesis system (Amersham), and the desired mutants were identified by DNA sequencing. The mutant fragments were returned to pCMV/Nc9, and the resulting constructs were named pCMV/p53-A mutants. To put the mutations into the retroviral construct, the *Hin*dIII/*Stu* I fragment of pLNL/XHCp53wt was substituted with the mutant fragments from pCMV/p53-A mutants. The mutation in each construct was confirmed by double-stranded DNA (dsDNA) sequencing before being transfected into COS cells.

Transfection and Selection of Stable Cell Lines. Cells (10^6) were seeded and transfected with a total of 20 μg of plasmid DNA per 10-cm plate as described (18). The day after transfection, cells were washed, the medium was changed, and G418 was added to the medium 1 day later. The medium was replaced every 3 or 4 days. Pools of G418-resistant colonies or single colonies were trypsinized and replated 14 days after transfection.

^{32}P-Labeling, Immunoprecipitation, and SDS Gel Electrophoresis. Cells were radiolabeled in 4 ml of phosphate-free DMEM supplemented with 10% dialyzed fetal calf serum and [^{32}P]orthophosphate (ICN) at a specific activity of 2.5 mCi/ml (1 Ci = 37 GBq) for 16 hr. Cell lysates were prepared

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Abbreviations: SV40, simian virus 40; DNA-PK, double-stranded DNA-dependent kinase; T antigen, large tumor antigen; dsDNA, double-stranded DNA; CMV, cytomegalovirus.

in RIPA buffer [0.15 M NaCl/1% (vol/vol) Nonidet P-40/0.1% (wt/vol) SDS/1% (wt/vol) sodium deoxycholate/10 mM sodium phosphate (pH 7.0)/1% Trasylol/50 mM leupeptin/1 mM dithiothreitol] and clarified by centrifugation at 20,000 × g for 30 min at 4°C. Immunoprecipitations were performed with hybridoma tissue culture supernatants containing p53-specific monoclonal antibodies, PAb122 (19) or PAb246 (20). Immunoprecipitates were separated by SDS/PAGE, as described (21).

Two-Dimensional Tryptic Phosphopeptide Mapping and Partial Cleavage of the Peptides with HCl. ^{32}P-labeled proteins were extracted from SDS/PAGE gels and oxidized as described (22). After trypsin digestion, peptides were separated on cellulose thin-layer plates by electrophoresis at pH 1.9 for 40 min at 1 kV in the first dimension and by chromatography in the second dimension (23). For partial cleavage of the peptides with HCl, the peptides were recovered from TLC plates and lyophilized. The peptides were then resuspended in 50 μl of 6 M HCl and incubated for 30 min at 110°C. After lyophilization, the cleaved products were dissolved in 5 μl of pH 3.5 buffer and loaded on TLC plates. Electrophoresis was carried out at 1 kV for 40 min.

HeLa Cell Lysate Preparation and Partial Purification of DNA-PK. HeLa cells were grown in ten 15-cm tissue culture dishes to near confluence (≈10^8 cells total) and harvested by trypsinization and centrifugation at 4°C. Cell extract was prepared by a single freeze–thaw cycle as described (24). DNA-PK was partially purified as follows. HeLa cell extract was centrifuged at 100,000 × g, and the pellet was dissolved in Hepes buffer (25 mM Hepes, pH 7.5/0.2 mM EDTA/0.5 mM KCl/10 mM $MgCl_2$). The material in the dissolved pellet was chromatographed on a 1-ml DEAE-cellulose DE52 column, as described (14). DNA-PK activity of the fractions was monitored by an *in vitro* assay, using p53 expressed in bacteria as substrate. The kinase active fractions were pooled, aliquoted, and stored in liquid nitrogen.

***In Vitro* Kinase Assay.** For the kinase assay, HeLa cell lysate was used as enzyme source. Immunoprecipitates containing ≈0.2 μg of p53 expressed in bacteria were resuspended in 20 μl of HeLa cell extract supplemented with 6 μl of KM solution (2 M KCl/10 mM $MgCl_2$) and 2 μl of 0.2 M EGTA per 100-μl cell lysate (25). [γ-^{32}P]ATP was added to a final concentration of 0.2 mM (specific activity of 4 mCi/nmol) immediately before the initiation of the kinase assay. For the kinase assay in which partially purified DNA-PK was used, the reaction mixture was composed of 10 μl of 2× kinase buffer (100 mM Hepes, pH 7.5/20 mM $MgCl_2$/4.0 mM EGTA/0.2 mM EDTA), 4 μl of purified kinase in Hepes/KCl buffer (25 mM Hepes, pH 7.5/0.2 mM EDTA/0.5 mM dithiothreitol/0.5 M KCl), and 6 μl of 0.25 mM [γ-^{32}P]ATP (specific activity of 10 mCi/nmol). Sonicated calf thymus DNA at a concentration of 10 μg/ml was added to the reaction mixture (when needed). The kinase assay was carried out for 10 min at 30°C.

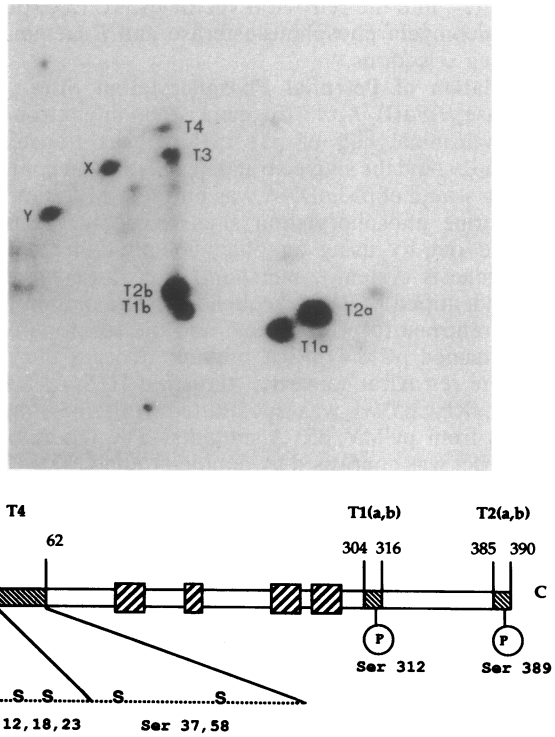

FIG. 1. Tryptic phosphopeptide map of wild-type mouse p53 expressed in COS cells. p53 protein was metabolically labeled with [^{32}P]orthophosphate and immunoprecipitated from cell extracts with monoclonal antibody PAb246, which is specific for wild-type mouse p53. The protein was extracted from an SDS/PAGE gel, oxidized, and digested with trypsin. Peptides were separated by electrophoresis in the first dimension (anode on left; cathode on right) and chromatography in the second dimension.

RESULTS

Phosphorylation of Mouse p53 *in Vivo*. Fig. 1 shows a tryptic phosphopeptide map of mouse p53. Wild-type mouse p53 was expressed in COS cells, radiolabeled with [^{32}P]orthophosphate, and immunoprecipitated from cell extracts with PAb246, a monoclonal antibody specific for wild-type mouse p53. Tryptic peptides were prepared as described in *Materials and Methods* and separated by electrophoresis in the first dimension and chromatography in the second dimension. It was shown previously (10, 26) that peptides T1a and T1b contain serine-312 and that peptides T2a and T2b contain serine-389. Peptide T4 (amino acids 28–62) contains two serine residues, one of which, serine-37, is phosphorylated *in vivo*. Peptide T3 (amino acids 1–27) contains five serine residues, one or more of which are phosphorylated.

The positions of peptides X and Y suggested that they might be multiply phosphorylated forms of peptide T3. We recovered the peptides from the TLC plate, partially cleaved them with HCl, and separated the products by electrophoresis, as shown in Fig. 2. Peptides T3, X, and Y produced similar cleavage products, supporting the idea that they were related. We plotted the predicted mobilities of the tryptic phosphopeptides, based on the molecular mass, the net

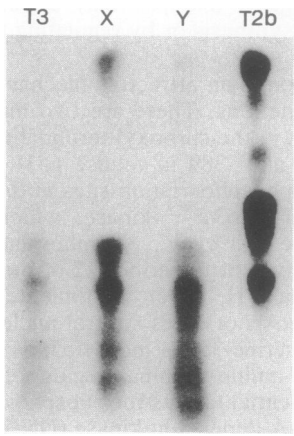

FIG. 2. Partial hydrolysis of tryptic phosphopeptides with HCl. Peptides T3, X, T2b, and Y were recovered from a TLC plate similar to that shown in Fig. 1 and partially hydrolyzed in 6 M HCl for 30 min at 110°C. The products were resolved by electrophoresis in pH 3.5 buffer at 1 kV for 40 min (anode at top; origin at bottom, out of figure).

charge, and the hydrophobicity of the peptides (27), as shown in Fig. 3. The predicted positions of peptide T3 with one (T3-1), two (T3-2), and three (T3-3) phosphoserine residues are very similar to the positions of T3, X, and Y in the authentic peptide map. The relatedness of the HCl cleavage products and the predicted positions of the peptides support the conclusion that T3, X, and Y are the same peptide with one, two, and three phosphoserine residues.

Amino-Terminal Phosphorylation Sites. To determine the phosphorylation sites in the amino-terminal region, we mutated the serine amino acid codons singly and in combinations. Loss of an acceptor site should result in a change in the tryptic peptide pattern—for example, loss of a phosphorylated peptide. We expressed the mutant proteins in COS cells and analyzed them by tryptic phosphopeptide mapping. Fig. 4 shows the maps of four mutants. Conversion of serine-12 to alanine did not change the pattern of phosphorylated peptides (Fig. 4*A*). Therefore serine-12 is not a phosphorylation site *in vivo*. Conversion of serine-9 to alanine (Fig. 4*C*) resulted in the loss of the triply phosphorylated peptide, T3-3, suggesting that serine-9 is a phosphorylation site *in vivo*. Conversion of serine-7 to alanine (Fig. 4*B*) resulted in the loss of the triply phosphorylated peptide, T3-3, suggesting that serine-7 is a phosphorylation site. There was also a decrease in the doubly phosphorylated peptide, T3-2, suggesting that if serine-7 is not phosphorylated another serine, such as the neighboring serine-9, is phosphorylated less efficiently. Conversion of serine-7, -9, and -12 to alanine (Fig. 4*D*) resulted in the loss of both the doubly and triply phosphorylated peptides, T3-2 and T3-3, further supporting the conclusion that serine-7 and -9 are phosphorylation sites *in vivo*. We observed new spots, migrating near peptide T4, in the maps of the serine-7 mutant and the triple mutant (Fig. 4 *B* and *D*), suggesting that mutation of serine-7 to alanine might result in phosphorylation at another site(s). We were unable to recover sufficient material to analyze these new spots further.

To identify the third phosphorylation site on peptide T3, we compared the tryptic peptide maps of the single mutant, serine-23 to alanine, and the double mutant, serine-18 and -23 to alanine residues (Fig. 5). The map of the single mutant (Fig. 5*D*) is identical to the map of the wild type (Fig. 5*A*), suggesting that serine-23 is not phosphorylated *in vivo*. The triply phosphorylated peptide, T3-3, is missing in the map of the double mutant (Fig. 5*C*), suggesting that serine-18 is the third phosphorylation site.

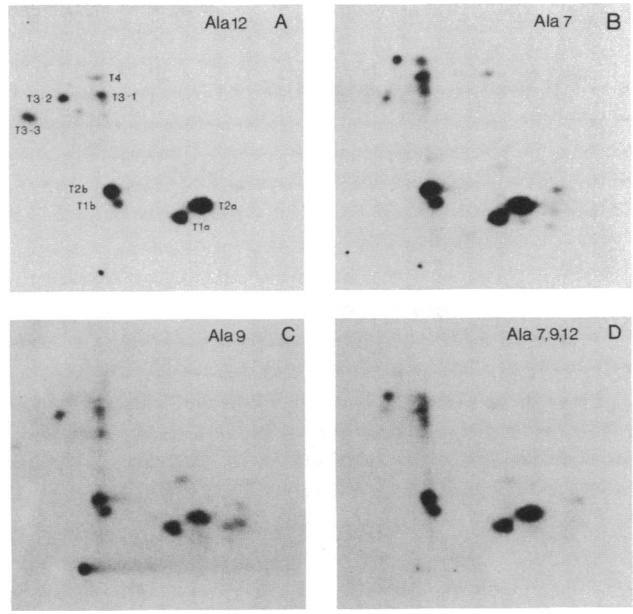

FIG. 4. Tryptic phosphopeptide maps of p53 serine-7, -9, and -12 phosphorylation site mutants. The maps were made as described in Fig. 1. (*A*) Serine-12 changed to alanine. (*B*) Serine-7 changed to alanine. (*C*) Serine-9 changed to alanine. (*D*) Serine-7, -9, and -12 changed to alanine residues.

To identify the phosphorylation site in peptide T4, we analyzed the serine-58 to alanine mutant. As shown in Fig. 5*B*, phosphorylation of peptide T4 was not affected by mutating serine-58 to alanine, suggesting that serine-58 is not an *in vivo* phosphorylation site. This observation implies that the only other serine in peptide T4, serine-37, is the phosphorylation site *in vivo*. This conclusion is consistent with the earlier prediction that serine-37 is phosphorylated *in vivo* (10).

In summary, serine-7, -9, -18, and -37 in the amino-terminal region of mouse p53 are phosphorylated *in vivo*; serine-12,

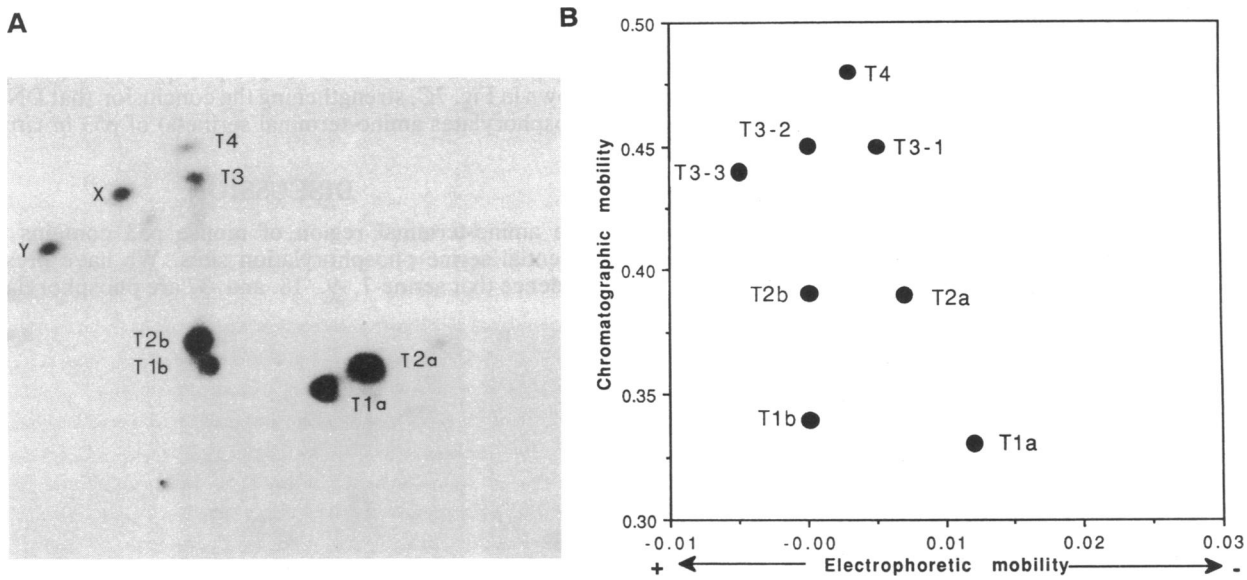

FIG. 3. (*A*) Authentic tryptic phosphopeptides of p53 radiolabeled *in vivo*. (*B*) Predicted mobility of tryptic phosphopeptides of mouse p53 separated in two dimensions. The predicted positions of tryptic phosphopeptides of p53 were calculated as described (27). The electrophoretic mobility is expressed as a charge-to-mass ratio for each peptide. The chromatographic mobility is an average of the mobilities of the individual amino acids of each peptide relative to a standard. Peptides T3-1, -2, and -3 are the amino-terminal peptide (amino acids 1–27) with one, two, and three phosphates.

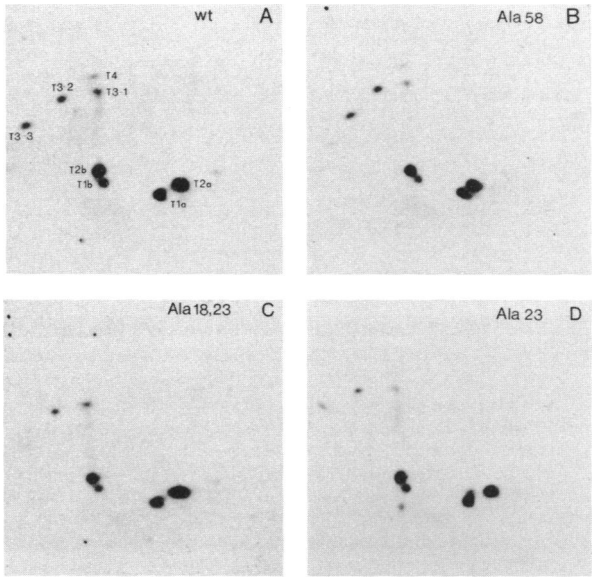

FIG. 5. Tryptic phosphopeptide maps of p53 serine-18, -23, and -58 phosphorylation site mutants. The maps were made as described in Fig. 1. (*A*) Wild-type mouse p53. (*B*) Serine-58 changed to alanine. (*C*) Serine-18 and -23 changed to alanine residues. (*D*) Serine-23 changed to alanine.

-23, and -58 are not phosphorylated; phosphorylation of serine-7 appears to facilitate phosphorylation of serine-9.

Phosphorylation of Amino-Terminal Sites by DNA-PK. DNA-PK phosphorylates baculovirus-expressed mouse p53 *in vitro* (14). It was suggested that serine-7 and -9 might be the targets for DNA-PK on the basis of the kinase recognition motif. To determine whether this kinase indeed phosphorylates amino-terminal sites in p53, we expressed p53 in bacteria, as described (13), and phosphorylated the bacterial p53 protein in an immune complex kinase assay with partially purified DNA-PK prepared from HeLa cells. The bacterial p53 was precipitated efficiently by PAb246 and less efficiently by PAb240, indicating that the protein was in a wild-type conformation (data not shown). Fig. 6 shows the results of the kinase assays using a HeLa cell lysate (*A*) and partially purified DNA-PK (*B*), in the presence and absence of dsDNA. The phosphorylation of several HeLa cell proteins, including hsp90 and p45, was stimulated in the presence of dsDNA, as reported (Fig. 6*A*, lanes 1 and 2) (25). Phosphorylation of bacterial p53 was also stimulated up to 10-fold in the presence of dsDNA (Fig. 6, lanes 1 and 2). Phosphorylation in the presence of a 20-fold excess of nonradioactive GTP over radioactive ATP did not decrease incorporation of radioactivity into p53, indicating that GTP did not compete with ATP as a phosphate donor (Fig. 6*B*, lane 3). Phosphorylation was inhibited by heparin (Fig. 6*B*, lane 4).

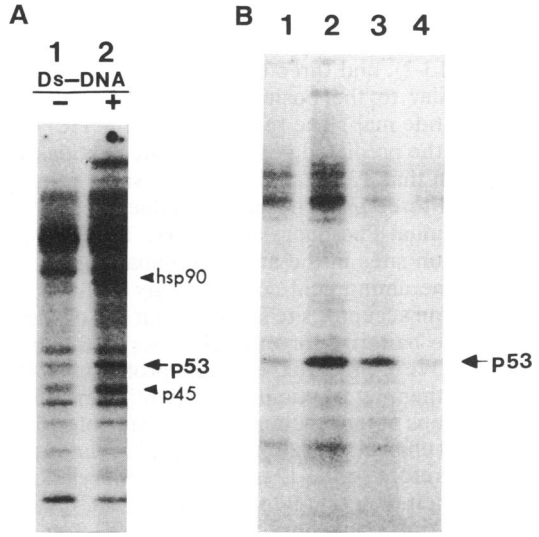

FIG. 6. Phosphorylation of mouse p53 expressed in bacteria by DNA-PK. Mouse p53 was immunoprecipitated from bacterial cell lysates by PAb246. Kinase assays were performed as described in *Materials and Methods*. (*A*) Phosphorylation by HeLa cell lysate in the absence (lane 1) or presence (lane 2) of calf thymus DNA. (*B*) Phosphorylation by partially purified DNA-PK in the absence of DNA (lane 1), in the presence of DNA (lane 2), or in the presence of DNA plus nonradioactive GTP (lane 3) or 0.1 μM heparin (lane 4).

We analyzed the bacterial p53 phosphorylated by DNA-PK by two-dimensional tryptic peptide mapping, as shown in Fig. 7. Fig. 7*A* shows the pattern of bacterial p53 phosphorylated by DNA-PK *in vitro*; Fig. 7*B* shows the pattern of wild-type p53 phosphorylated *in vivo*. Serine-312 and -389, which are phosphorylated *in vivo*, were not phosphorylated *in vitro*. The migration of the major phosphorylated peptide(s) from the *in vitro* reaction was similar to that of the amino-terminal peptide, T3, of the protein phosphorylated *in vivo*. The multiply phosphorylated peptides, T3-2 and T3-3, were not present in the *in vitro* reaction, and a new minor peptide was present. Because of the impurity of the kinase preparation, it might be expected that other kinases or phosphatases could be acting on the p53 protein in the *in vitro* reaction. When the *in vivo* and *in vitro* phosphorylated peptides of p53 were mixed, the upper spot of the *in vitro* phosphorylated p53 comigrated with peptide T3 of p53 phosphorylated *in vivo*, as shown in Fig. 7*C*, strengthening the conclusion that DNA-PK phosphorylates amino-terminal serine(s) of p53 *in vitro*.

DISCUSSION

The amino-terminal region of mouse p53 contains seven potential serine phosphorylation sites. We have presented evidence that serine-7, -9, -18, and -37 are phosphorylated in

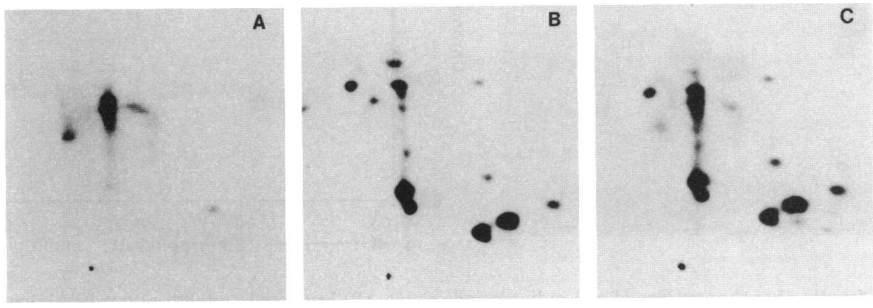

FIG. 7. Tryptic phosphopeptide map of mouse p53 phosphorylated by DNA-PK *in vitro*. The map was prepared as described in *Materials and Methods*. (*A*) Wild-type p53 phosphorylated *in vitro*. (*B*) Wild-type p53 phosphorylated by DNA-PK *in vivo*. (*C*) Mixture of *in vivo* and *in vitro* phosphorylated peptides of p53.

mouse p53 expressed in COS cells, whereas serine-12, -23, and -58 are not. The other prominent phosphorylation sites in mouse p53 are serine-312 and -389. The amino-terminal phosphoserine residues are contained in two tryptic phosphopeptides, T3 and T4. The experiments reported here do not allow us to estimate the stoichiometry of *in vivo* phosphorylation. However several lines of evidence support the conclusion that a single molecule of the amino-terminal tryptic peptide, T3, can contain up to three phosphorylated serines. Peptides T3, X, and Y, treated with HCl, produced partial cleavage products with similar electrophoretic mobilities, suggesting that T3, X, and Y represent the same peptide with one, two, and three phosphate groups. The predicted mobility of peptide T3 containing two and three phosphate groups corresponds well to the actual mobilities of X and Y. Serine-7, -9, and -18, which are contained in peptide T3, are phosphorylated *in vivo*, and the loss of peptides X and Y in mutant p53s lacking serine-7 and -9 phosphorylation sites is consistent with phosphorylation of T3 on up to three serine residues.

Mutation of serine-7 to alanine resulted in loss of the triply phosphorylated peptide, Y, and a decrease in the amount of the doubly phosphorylated peptide, X, suggesting that phosphorylation of serine-7 may facilitate the phosphorylation of serine-9. There are at least two possible explanations for this observation. Serine-7 and -9 could be phosphorylated by the same kinase, which phosphorylates serine-7 first and then phosphorylates the neighboring serine-9. Serine-7 is contained in a recognition sequence for DNA-PK, ESQ, which is identical to a recognition sequence in hsp90 (14). Serine-9 is contained in a similar, but not identical, element, QSD, which might allow serine-7 to be recognized preferentially. A second possible explanation is that phosphorylation of serine-7 is important for the recognition of serine-9 by the kinase, which phosphorylates it. Recognition of phosphorylation sites in rabbit muscle glycogen synthase by glycogen synthase kinase 3, for example, requires phosphorylation of a serine residue in the recognition sequence by another protein kinase (28).

DNA-PK was first identified as a kinase that phosphorylated hsp90 (25). Two threonine residues are phosphorylated in the sequence, PEETQTQDQPM, in hsp90 (29). A search for proteins with the sequence elements -E-(T/S)-Q- or -Q-(T/S)-Q- showed that several nuclear DNA-binding proteins, including the transcription factors Sp-1, Oct-1, and Oct-2; topoisomerases I and II; SV40 T antigen; and p53, contained these elements (14). Purified SV40 T antigen and p53 are phosphorylated by DNA-PK *in vitro*. It was noted previously that serine-7 (ESQ) and -9 (QSD) of p53 resemble hsp90 phosphorylation sites (14). The conclusion from tryptic phosphopeptide analysis that DNA-PK appears to phosphorylate serine residues in the amino-terminal region of p53 is consistent with phosphorylation of serine-7 and -9. Although p53 is a good substrate for DNA-PK *in vitro*, it is not known whether p53 is phosphorylated by DNA-PK *in vivo*. DNA-PK has been described so far only in HeLa cells, which lack p53. It is possible that another kinase(s) is responsible for amino-terminal phosphorylation of p53 in mouse cells or that DNA-PK is present, but appropriate conditions for assaying it have not yet been established.

The wild-type p53 protein exhibits several biochemical and biological activities, including transactivation, DNA binding, inhibition of SV40 origin-driven DNA replication, and inhibition of cell growth, any of which might be regulated by phosphorylation. It will be interesting to test the effects of mutations in phosphorylation sites on these activities.

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