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Mapping of phosphomonoester and apparent phosphodiester bonds of the oncogene product p53 from simian virus 40-transformed 3T3 cells

(protein phosphorylation/oncogene product/protein-bound nucleotide/radiochemical protein sequencing/simian virus 40 tsA mutant)

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ABSTRACT **The oncogene product p53, isolated from SV3T3 cells where it forms a complex with simian virus 40 large tumor antigen (T antigen) in the nucleus, has been found to be phosphorylated at at least four distinct sites on the 390 amino acid protein. Separation of tryptic phosphopeptides has permitted identification of two sites as Ser-312 and Ser-389, and permitted analysis of the types of phosphate bonds. The peptide containing Ser-312 separates electrophoretically into three charged forms; two are resistant to dephosphorylation by both alkaline phosphatase and alkaline hydrolysis, suggesting a phosphodiester. The carboxyl-terminal phosphopeptide containing Ser-389 was alkaline phosphatase-resistant and liberated four ribonucleoside monophosphates upon base or RNase hydrolysis, suggesting that Ser-389 may be covalently linked to RNA. Phosphorylation of Ser-389 decreased markedly at the nonpermissive temperature in simian virus 40 tsA58-transformed cells, indicating a dependence on native T antigen function and a possible role in transformation by T antigen. Two additional phosphorylation sites, one involving serine and one involving threonine, probably reside in the amino-terminal segment of p53 and appear to be peptide-phosphate monoesters.**

The oncogene product p53 is found elevated in cells transformed by a variety of agents, including the DNA tumor viruses simian virus 40 (SV40), adenovirus type 2, and Epstein–Barr virus, RNA tumor viruses such as Abelson leukemia virus, chemical carcinogens, ionizing radiation, and it is also increased in various spontaneous tumors (reviewed in ref. 1). Recently, mouse p53 was shown to immortalize primary rat embryo fibroblast cells transfected with its cloned gene (2) and to render the cells tumorigenic when cotransfected with *ras* gene of Harvey sarcoma virus (3, 4). p53 forms complexes with both SV40 large tumor antigen (T antigen) (5) and the adenovirus E1B 58-kDa tumor antigen (6). The increased concentration of p53 in transformed cells is thought to play a role in establishment of the transformed phenotype. p53 may also play a role in normal cell cycle control and in cell differentiation (7, 8).

p53, like many tumor antigens, is a phosphoprotein (1, 9). Although many enzymatic functions are modulated through phosphorylation (10), the role of phosphorylation in modulating the function of nuclear products of oncogenes is unclear. The availability of the complete mouse p53 amino acid sequence (11, 12), deduced from cloned genes, has allowed us to map and characterize individual phosphorylation sites that correlate with biochemical changes, such as binding to SV40 T antigen.

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MATERIALS AND METHODS

Preparation of p53. Mouse cells transformed by wild-type SV40 and by the temperature-sensitive A locus (tsA) mutant, tsA58, were obtained from G. Khoury and P. Tegtmeyer, respectively. The hybridoma line PAb416, used in all immunoprecipitations, was obtained from E. Harlow. Cells were labeled with [^{32}P]orthophosphate, and in some cases, a tritiated amino acid was used, as indicated in the text. Extracts containing the placental ribonuclease inhibitor RNasin (600 μg/ml) (Promega Biotec, Madison, WI) were immunoprecipitated and fractionated by NaDodSO$_4$/PAGE, as described (13). p53 was electroeluted from gels after addition of protease- and nuclease-free bovine serum albumin as carrier (Bethesda Research Laboratories). All tubes were siliconized and sterilized.

Peptide Separation and Sequence Analysis. Eluted p53 was precipitated with ice-cold trichloroacetic acid (20% vol/vol for 1 hr at 0°C), extracted with ethanol/ether at 1:1 and 1:2 to remove NaDodSO$_4$, and the pellets were air dried. After performic acid oxidation at 0°C for 2 hr, the lyophilized protein was dissolved in 400 μl of 50 mM NH_4HCO_3 (pH 8) and digested at 37°C with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington), first for 2 hr at a trypsin/carrier ratio of 1:20 (wt/wt), and then for 4 hr after addition of a second equal portion of trypsin. After lyophilization, the peptides were dissolved in 0.1% trifluoroacetic acid (Pierce) by heating for 10 min at 37°C. After centrifugation at 12,000 × g to remove insoluble material, the peptides were applied to a 3.9 mm × 30 cm μBondapak C18 reverse-phase column (Waters Associates) equilibrated with 0.1% trifluoroacetic acid (pH2). Peptides were eluted with a nonlinear gradient of 0.1% trifluoroacetic acid/80% acetonitrile/20% water, at a flow rate of 1 ml/min, and 1-ml fractions were collected. Peptides labeled with ^{32}P were detected by Cerenkov radiation or scintillation counting, and pooled peak fractions were suspended in 5% acetate/0.5% pyridine, pH 3.5, and electrophoresed on Whatman 3 MM paper at 200 mA (14). Appropriate pooled fractions were dissolved in 20% formic acid and applied to a Beckman 890C protein sequencer with 3 mg of Polybrene and 2 mg of apomyoglobin for analysis as described (15).

Analyses of Phosphate Bonds. Phosphorylated amino acids were identified by two-dimensional thin layer electrophoresis, after acid hydrolysis, as described (13). For base hydrolysis, phosphopeptides were dissolved in 50 μl of 0.3 M KOH, and incubated for 20 hr at 37°C. Alkaline phosphatase digestion was done in 20 μl of 20 mM Tris·HCl (pH 8)/1 mM NaEDTA/1 unit of alkaline phosphatase (Boehringer Mannheim) for 1 hr at 37°C.

Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen.

RESULTS

p53 Tryptic Phosphopeptides. To determine the minimum number of phosphorylation sites in p53, ^{32}P-labeled p53 was digested with trypsin and individual tryptic peptides were resolved by reverse-phase HPLC (Fig. 1). Four major radioactive components were observed and designated P1–P4 in order of increasing hydrophobicity. P1 consisted of material not retained by the column and, therefore, is likely to consist of multiple components. P2 eluted heterogeneously, sloping toward the flow through (P1). Peak 3 (P3a) eluted as a sharp peak with a small shoulder (P3b), which is probably a related peptide (see below). The last component was a group of three peaks (P4a, -b, and -c) believed to be related and thus considered collectively. These peaks were not obtained in a sham digest of p53.

To determine whether the peaks consisted of multiple species, each was further fractionated by high voltage paper electrophoresis (Fig. 2). All except P4 yielded two or more phosphorylated species: P4 did not migrate from the origin during electrophoresis at pH 3.5. P3a yielded 3 differently charged species (designated 3-1, 3-2, and 3-3). The shoulder, P3b, consisted of three species of precisely the same mobilities as those of P3a. P2 yielded one major species (2-1) that remained near the origin, and a smear of phosphorylated material migrating toward the anode (2-2). P1 gave three major phosphate-containing species (1-1, 1-2, and 1-3).

Each of the species shown in Fig. 2 was analyzed for phosphoamino acid content by two-dimensional thin layer analysis after acid hydrolysis (summarized in Table 1). All except P4 yielded only phosphoserine (*P*-Ser) and inorganic phosphate (P_i). P4 (a–c pooled) gave *P*-Ser and a lesser amount of phosphothreonine (*P*-Thr). We have shown previously that intact p53 from SV3T3 cells contains ≈92% *P*-Ser and 8% *P*-Thr (13).

Sequence Identification of p53 Tryptic Phosphopeptides. To identify precisely amino acid residues that become phosphorylated, SV3T3 cells were labeled with $^{32}PO_4$ and one tritiated amino acid (at a time). The double-labeled peptides were applied to an automated protein sequencer, and the release of label after each Edman cycle was monitored by scintillation counting. P2 has thus been identified as the carboxyl-terminal tryptic peptide Lys-384–Asp-390, based on the release of tritium in residue 1 from [^{3}H]lysine-labeled P2 (Fig. 3), residue 3 from [^{3}H]glycine-labeled P2 (data not shown), residue 4 from [^{3}H]proline-labeled P2 (Fig. 3), and ^{32}P in residue 6 from $^{32}PO_4$-labeled P2 (Fig. 3). Release of phosphate at residue 6 is consistent with phosphorylation of

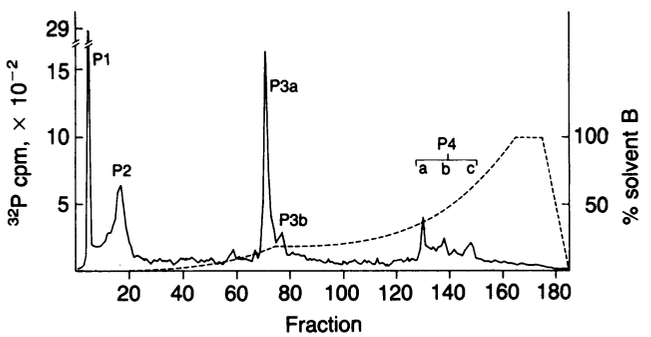

FIG. 1. Reverse-phase HPLC profile of p53 tryptic phosphopeptides. SV3T3 cells were radiolabeled for 18 hr in phosphate-free Dulbecco's modified Eagle's medium containing 10% dialyzed calf serum, 8% complete medium, and [^{32}P]orthophosphate at 1.2 mCi/ml (1 Ci = 37 GBq) (5 ml per plate). p53 tryptic peptides were injected into a C18 reverse-phase HPLC column preequilibrated with 0.1% trifluoroacetic acid, pH 2.1 (solvent A) and eluted with a nonlinear gradient (dashed line) increasing in solvent B (0.1% trifluoroacetic acid in 80% acetonitrile/20% H_2O). Approximately 50,000 cpm were injected. $^{32}PO_4$-labeled peptides were detected by Cerenkov counting, and are numbered in order of increasing hydrophobicity.

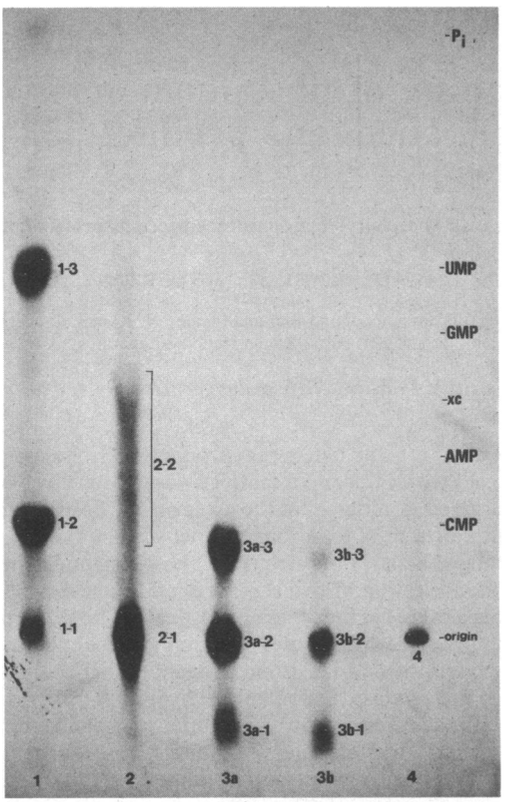

FIG. 2. High-voltage paper electrophoresis of ^{32}P-labeled HPLC peaks. $^{32}PO_4$-labeled p53 from SV3T3 cells was trypsinized and fragments were resolved by HPLC exactly as described in Fig. 1, except that $^{32}PO_4$ was used at a concentration of 2.4 mCi/ml (5 ml per plate). Labeled peaks were redissolved in 1% acetic acid and applied to 3 MM Whatman chromatography paper for electrophoresis (≈200 mA) in 0.5% pyridine/5% acetate, pH 3.5 (14). Phosphorylated species were numbered from the cathodal (bottom) to anodal (top) direction. Position of the 3′ ribonucleotide standards, the origin, and the xylene cyanol dye (xc) are indicated.

Table 1. Phosphoamino acid analysis of $^{32}PO_4$-labeled peptides

Phosphopeptide no.	*P*-Ser	*P*-Thr	*P*-Tyr
1-1	+	–	–
1-2	+	–	–
1-3	ND		
2-1	+	–	–
2-2	+	–	–
3a-1	+	–	–
3a-2	+	–	–
3a-3	+	–	–
3b-1	+	–	–
3b-2	+	–	–
3b-3	+	–	–
4a, -b, -c*	+	+	–

The spots shown in Fig. 2 were eluted in 50 mM NH_4HCO_3, lyophilized, and hydrolyzed 2 hr at 110°C under vacuum in 6 M HCl, and applied to thin-layer cellulose sheets, then separated by two-dimensional electrophoresis at pH 1.9 (first dimension) in acetic acid/formic acid/H_2O (25:78:897) and at pH 3.5 (second dimension) in pyridine/acetic acid/H_2O (10:100:1890).
*Peaks 4a, 4b, and 4c were pooled for analysis.

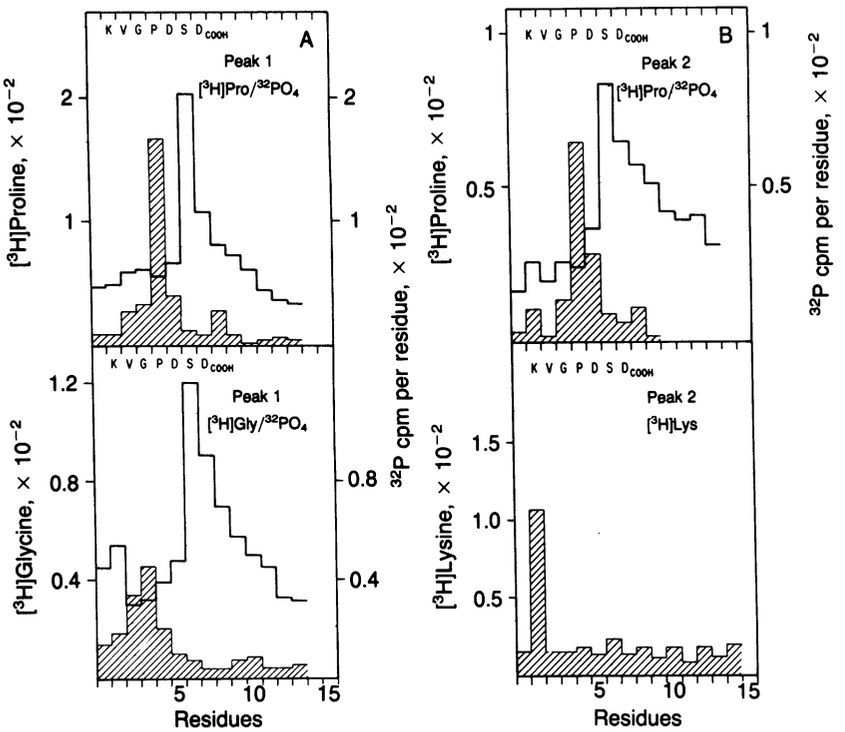

FIG. 3. Amino acid sequencing. SV3T3 cells were labeled with one tritiated amino acid for 18 hr, starved in PO_4-free Dulbecco's modified Eagle's medium (DMEM)/10% dialyzed calf serum for 1 hr, and pulsed with $^{32}PO_4$ at 1.8 mCi/ml (3.5 ml per plate) in PO_4-free DMEM for 3.5 hr. p53 was treated as described in Fig. 1. (*A*) Pooled fractions from peak 1 were fractionated by paper electrophoresis, and the peptide corresponding to spot 1-1 in Fig. 2 was eluted, dried, and applied to a Beckman 890C sequencer. Yields of radioactivity after each Edman degradation cycle are shown for both ^3H (cross-hatched bars) and $^{32}PO_4$ (heavy line). The predicted amino acid sequence of the peptide is shown above each panel in the single-letter amino acid code. Radioactivity applied to the sequencer: [^{3}H]proline, 1035 cpm (*Upper*); [^{3}H]glycine, 774 cpm (*Lower*). (*B*) The peptide corresponding to spot 2-1 in Fig. 2 was isolated and sequenced exactly as in *A*. Radioactivity applied: [^{3}H]proline, 633 cpm (*Upper*); [^{3}H]lysine, 560 cpm (*Lower*); [^{3}H]glycine, 370 cpm (data not shown).

Ser-389. No other site in the predicted sequence of p53 can be accommodated by these results; however, careful analysis suggests that P2 probably contains a smaller quantity of a second peptide, Val-271–Arg-277, which does not contain serine or threonine and, thus, cannot be phosphorylated.

The species 1-1 from peak 1 was found also to have glycine at residue 3, proline at residue 4, and phosphate at residue 6 (Fig. 3), indicating that P1 also contains a phosphorylated form of the carboxyl-terminal tryptic peptide; however, not unexpectedly, analysis of the released radioactivity reveals the presence of several other nonretained peptides.

P3 was found to have leucine at residue 2; proline at residues 3, 10, and 11; cysteine at residue 5; and phosphate at residue 9 (Fig. 4). The presence of proline at residues 3, 10, and 11 indicates P3 contains the tryptic peptide, Ala-304–Lys-316 or Ala-304–Lys-317. Although residues 310 and 312 are predicted to be serine, only residue 312 is phosphorylated. Since sequence analysis of P3 not further fractionated by high-voltage paper electrophoresis provided evidence for only one phosphorylated peptide, we believe that the three differently charged species observed by electrophoretic analysis (Fig. 2) result from different charge modifications of this peptide. Lys-316 is immediately followed by the sequence Lys-317–Lys-318–Pro-319–Leu-320–. We suggest that P3a ends with Lys-316 because, in our experience, trypsin cleaves most efficiently after the first of several consecutive basic amino acids (16). Furthermore, lysine-proline bonds are relatively resistant to trypsin cleavage. Therefore, P3b is likely to result from cleavage after Lys-317 or Lys-318, instead of Lys-316. In the trifluoroacetic acid/acetonitrile solvent system, the data of Sasagawa *et al.* (17) indicate that lysine provides a small net increase in peptide retention on silica-based reverse-phase columns.

P4 is rather hydrophobic. We have not yet identified this peptide by direct sequencing, but analysis of amino acid-labeled tryptic digests and theoretical retention times for p53 tryptic peptides suggest that P4 may correspond to one (or both) of two large peptides (Leu-28–Arg-62; Val-63–Lys-98) from the amino terminus of p53.

The Carboxyl-Terminal p53 Tryptic Peptide Is Associated with Ribonucleotides. Each of the phosphorylated species shown in Fig. 2 was eluted, treated with base or with alkaline phosphatase, and the products were analyzed by high-voltage paper electrophoresis. Fig. 5 shows the results of base hydrolysis. P4 phosphate was liberated by base, but was resistant to 1 M HCl and to treatment by hydroxylamine (data not shown), as expected for phosphate monoesters of serine and threonine (18, 19). In contrast, digestion of P3 with base yielded significant P_i, but part of the radioactivity was resistant to digestion. The charged species 3-3 was found to be labile to alkaline phosphatase, while 3-1 and 3-2 were resistant (data not shown). These results are consistent with our conclusion that 3-1, 3-2, and 3-3 probably represent modified forms of the same peptide, and that at least part of the Ala-304–Lys-316 peptide-bound phosphate may be in the form of a phosphate diester.

Base hydrolysis of the carboxyl-terminal tryptic phosphopeptide (species 2-1 and 2-2) yielded some P_i and material with the electrophoretic mobility of each of the four ribonucleoside monophosphates included as standards during electrophoresis. Hydrolysis of 2-1 and 2-2 with 7 μg of RNase A and 1 unit of RNase T2 in 50 mM sodium acetate (pH 6.0) for 1 hr at 37°C also yielded four ribonucleoside monophosphates (data not shown). A substantial proportion of P2 was also insensitive to alkaline phosphatase (data not shown), as expected of phosphodiester bonds (18). P1 was also largely resistant to hydrolysis by base and alkaline phosphatase, as might be expected if a significant proportion of P1 were derived from the carboxyl-terminal tryptic peptide. Base hydrolysis of species 1-1 yielded some P_i and material with the mobility of 1-2 and 1-3. Hydrolysis of 1-2 yielded some P_i and material with mobility of 1-3. However, both 1-2 and 1-3 were largely resistant to base hydrolysis. 1-2 and 1-3 have mobilities similar to CMP and UMP, respectively, but as noted above, acid hydrolysis of 1-2 yielded *P*-Ser. Since the pH of individual hydrolyses was always checked, the resistance of P1, P2, and P3 to base and alkaline phosphatase hydrolysis is unlikely to be due to inappropriate digestion conditions.

Efficient Phosphorylation of p53 Ser-389 Requires Functional T Antigen. To examine the possibility that the phosphorylation of p53 might be influenced by SV40 T antigen, 3T3

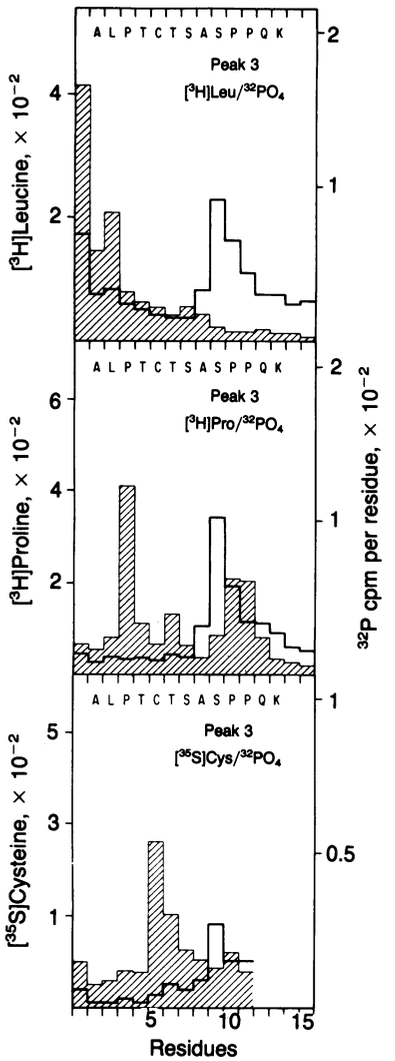

FIG. 4. Amino acid sequencing. p53 was prepared and pooled fractions from peak 3a were sequenced as described in Fig. 3. Radioactivity applied: [^{3}H]leucine, 927 cpm (*Upper*); [^{3}H]proline, 3717 cpm (*Middle*); [^{35}S]cysteine, 2970 cpm (*Lower*).

cells transformed with a temperature-sensitive SV40 A locus mutant, tsA58, were labeled for 5 hr with $^{32}PO_4$ at the permissive temperature (32°C), or for 5 hr, 24 hr after a shift to the nonpermissive temperature (39.5°C). As can be seen from the HPLC profiles of ^{32}P-labeled p53 tryptic peptides in Fig. 6, increasing the temperature to 39.5°C caused a dramatic reduction of radioactivity in P2, the carboxyl-terminal peptide, relative to other components. In control experiments (not shown), p53 from wild-type-transformed SV3T3 gave the same profile of ^{32}P-labeled tryptic peptides at both temperatures. Note that the apparent shift of label among the components of P4 probably resulted from small differences in digestion conditions, as such differences were observed in different preparations.

DISCUSSION

p53 is a member of the nuclear oncogene family that also includes c-*myc*, adenovirus EIA, and polyoma T antigens. Although data exist to support a role for phosphorylation in modulation of enzyme function (10), little is known about the effect of phosphorylation on the function of nuclear oncogene products. Except for SV40 T antigen (20, 21), none of the phosphorylation sites of these proteins has been unambiguously mapped. To understand the role of phosphorylation

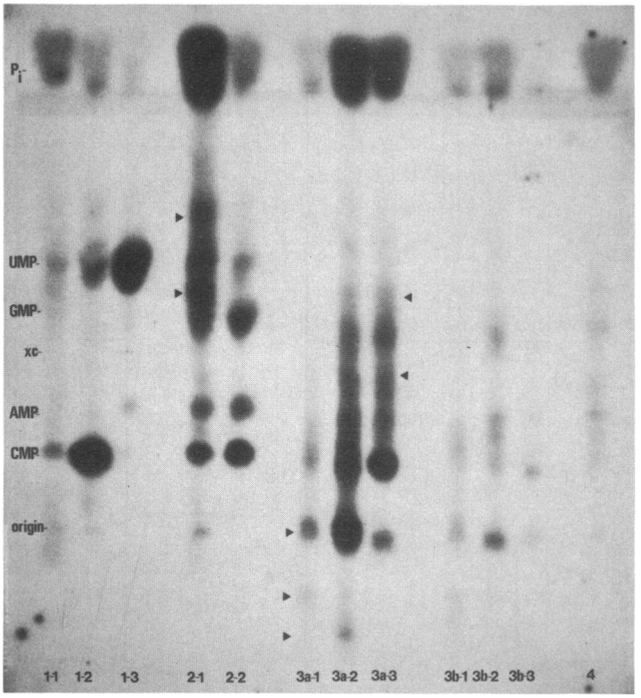

FIG. 5. Alkaline hydrolysis of p53 phosphopeptides separated by HPLC and high-voltage paper electrophoresis. Each phosphorylated species in Fig. 2 was eluted from 3 MM paper in 25 mM NH_4HCO_3, lyophilized, hydrolyzed for 20 hr at 37°C with 50 μl of 0.3 M KOH, and electrophoresed again as described in Fig. 2. Arrowheads indicate some of the major phosphorylated species that do not comigrate with any of the ribonucleotide standards. xc, Xylene cyanol dye.

on the biological activity of p53, we have attempted to map its phosphorylation sites precisely. Tryptic phosphopeptides of p53 prepared by immunoprecipitation and $NaDodSO_4$/PAGE were resolved into four phosphate-containing peaks

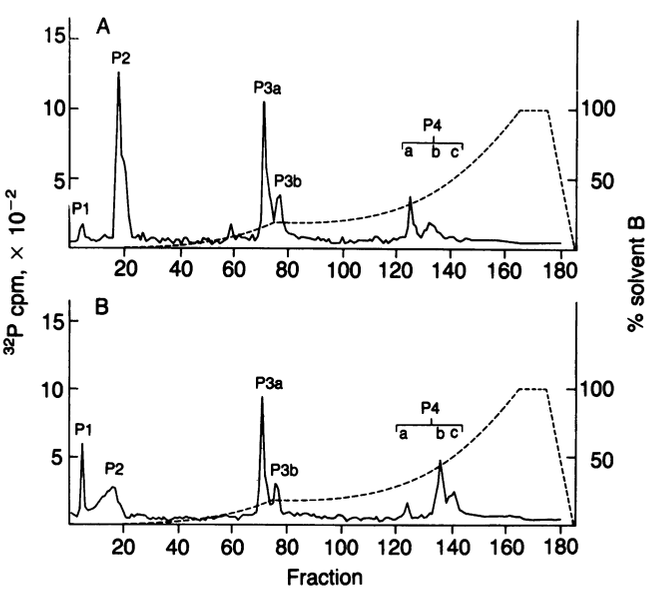

FIG. 6. Effect of *tsA* mutation on phosphorylation pattern of p53. p53 was prepared as described in Fig. 1 from mouse cells transformed by a SV40 temperature-sensitive mutant (tsA58) and maintained at either 32°C (*A*) or 39.5°C (*B*), trypsinized under identical conditions, and the fragments were resolved as described in Fig. 1. $^{32}PO_4$-labeled peptides were detected by scintillation counting. Approximately 13,000 cpm were injected for each sample.

by reverse-phase HPLC, and these were further fractionated by high-voltage paper electrophoresis. All phosphorylated species examined after high-voltage paper electrophoresis purification were shown to be bona fide phosphopeptides by phosphoamino acid analysis, and to contain *P*-Ser. Peak 4a–c also contained *P*-Thr. The phosphorylated amino acids in peaks 2 and 3a were identified by Edman sequencing, as Ser-389 and Ser-312, respectively. Phosphate on Ser-389 was resistant to alkaline phosphatase digestion, indicating that the phosphate was not present exclusively as a monoester (18). Treatment with base (KOH), or RNase, liberated four ribonucleoside monophosphates, inorganic phosphate, plus resistant phosphate. The data suggest that Ser-389 contains phosphate, at least in part, as a phosphodiester linkage to RNA. Although the combination of immunoprecipitation, $NaDodSO_4$/PAGE, HPLC, and paper electrophoresis might be expected to separate contaminant RNA from peptides, we are pursuing additional evidence for covalent binding to RNA. Recent results indicate that RNase digestion of the putative RNA-linked peptide renders it more hydrophobic (unpublished data). Both cellular and viral proteins have been found covalently linked to RNA (22, 23) and DNA (24, 25). However, except for a single report (26) that ribonucleotides may be associated with SV40 T antigen, no rigorous evidence is available to support the existence of nucleic acids covalently linked to products of oncogenes.

The nature of the phosphate bonding to Ser-312 remains unknown. Although three different forms of the peptide containing Ser-312 were detected by paper electrophoresis, only one had phosphate labile to alkaline phosphatase. Treatment of the phosphopeptide containing Ser-312 with base resulted in a complex mixture of resistant products. The results are consistent with the possible presence of a phosphodiester bond. In contrast, the most hydrophobic phosphopeptides (peaks 4a–c), which liberated *P*-Ser and a small amount of *P*-Thr, appear to contain only phosphomonoester bonds. The phosphates were tentatively mapped to the amino terminus, between Leu-28 and Lys-98.

Notably, Ser-389 and Ser-312, as well as adjoining sequences, are conserved in mouse and human p53 (27), implying that p53 from divergent species may be phosphorylated at similar positions. These sites also lie near, or within, regions of limited sequence homology between p53 and c-*myc* (28). Since c-*myc* is a phosphoprotein (29), it may be phosphorylated on similar sites as p53.

Temperature-sensitive mutations (tsA) in SV40 T antigen exist that render T antigen defective in both replication and transformation (30) and that inhibit complex formation with p53 at the nonpermissive temperature (31, 32). We have shown that p53 from cells transformed by a tsA mutant of SV40 exhibits markedly decreased phosphorylation of Ser-389 (but no other sites) at the nonpermissive temperature. Although we have not yet studied the relative rates of ^{32}P incorporation or turnover in individual sites at the nonpermissive temperature, the data suggest that phosphorylation of at least one site of p53 is dependent on native T antigen function, and that it may play a role in transformation by T antigen. The fact that phosphorylation of a site on p53 depends on the integrity of T antigen and that maximally phosphorylated T antigen is found in complexes with p53 (31) supports the notion that T antigen and p53 may operate through a concerted reaction involving phosphorylation. Ser-389 liberates ribonucleotides and may therefore be linked to RNA. We are currently determining whether the decrease in phosphorylation of Ser-389 reflects a reduction, or complete absence, of associated ribonucleotides, and whether or not this site is phosphorylated in cells transformed by agents other than SV40.

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