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Altered Phosphorylation of Free and Bound Forms of Monkey p53 and Simian Virus 40 Large T Antigen during Lytic Infection

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We have identified the phosphorylation sites in monkey p53 as well as specific changes in the phosphorylation state of free and complexed forms of simian virus 40 (SV40) large T antigen (T) and monkey p53 isolated from SV40 lytically infected CV1 cells. Phosphopeptide analyses of free T and p53 (To and p53o) and complexed T and p53 (T+ and p53+) fractions indicated several quantitative increases in the specific phosphorylation of complexed forms of both proteins. The N terminus of monkey p53+ is phosphorylated at Ser-9, Ser-15, Ser-20, either Ser-33 or Ser-37, and at least one of Ser-90 to Ser-99. The C-terminal sites are Ser-315 and Ser-392. On comparing p53+ with p53o, we found that labeling of the two N-terminal phosphotryptic peptides encompassing residues 1 to 20 and 33 to 101 was increased fivefold and that Ser-315 was sevenfold more labeled than was Ser-392. When T+ was compared with To, the N-terminal peptide containing phosphorylation sites Ser-106 through Thr-124 was twofold more labeled, the peptide containing Ser-657 through Ser-679 was sixfold more labeled and contained up to four phosphorylated serine residues, and Ser-639 and Thr-701 appeared unchanged. Overall, T+ molecules appeared to contain 3.5 mol more of labeled phosphate than did To, with the N-terminal peptide appearing fully phosphorylated. The phosphopeptide patterns obtained for lytic T+ and To fractions were nearly identical to those found for wild-type SV40 T (stably complexed with mouse p53) and mutant 5080 T (defective for p53 binding) expressed in transformed C3H10T1/2 cells (L. Tack, C. Cartwright, J. Wright, A. Srinivasan, W. Eckhart, K. Peden, and J. Pipas, J. Virol. 63:3362–3367, 1989). These results indicate that increases in specific phosphorylation sites in both T+ and p53+ correlate with the association of T with p53. The enhanced phosphorylation state may be a consequence of complex formation between T and p53 or reflect an increased affinity of p53 for highly phosphorylated forms of T.

Simian virus 40 (SV40) large T antigen (T) is a multifunctional phosphoprotein that is required for productive viral infection, transformation of permissive cells, and tumor formation in animals. T regulates the expression of SV40 and specific cellular genes and directly participates in several steps during SV40 DNA replication. At least three biochemical activities of T are required for DNA synthesis: specific *ori* DNA binding and melting, ATPase, and DNA helicase activities (reviewed in references 8, 34, and 53).

The cellular phosphoprotein p53 forms complexes with T in SV40-infected and SV40-transformed cells (reviewed in references 15, 18, and 24). Normal p53 has a short half-life and is found in trace quantities in normal cells; however, it is stabilized by association with T and is normally present at high levels in cells expressing wild-type T. Mutations in p53 can lead to oncogenic activation of the protein. These altered p53 forms can immortalize cells, cause malignant transformation, and cooperate with the *ras* oncogene in the transformation of primary cells, and they frequently have extended half-lives. Normal p53 is involved in cell cycle regulation, participating in the transition from G_0 to G_1. While p53 has an antiproliferative or tumor suppressor activity (12, 25), normal p53 appears to be required for reentry of cycling cells into S phase and thus may have a positive role in growth regulation (9). Besides complex formation with SV40 T, p53 associates with other oncogenic viral antigens such as papillomavirus E6 and adenovirus E1B (24). p53 has several other biochemical activities, including binding to specific SV40 *ori*-related and cellular DNA sequences, transcriptional transactivation, and oligomerization (2, 11, 15, 21, 35).

Complex formation between SV40 T and monkey p53 (T+p53) has a major role in SV40-mediated cell transformation and affects a number of replication-related activities of T (reviewed in reference 24). These include ATPase and helicase activities, binding to SV40 *ori* DNA sequences, binding to DNA polymerase α, and in vitro SV40 DNA replication (13, 16, 22, 49, 51, 55). T+p53 complexes isolated from lytically infected cells were observed to bind efficiently to SV40 DNA sequences in the 21-bp repeat and SP1 region, in addition to binding to the normal T sites I and II at SV40 *ori* (51). The same 21-bp repeat transcriptional control region has been recently shown to bind purified wild-type p53 (2). However, formation of stable T+p53 complexes does not appear to be an absolute requirement for SV40 DNA replication (26).

T and murine p53 are both multiply phosphorylated at N- and C-terminal sites (28, 38, 41, 42). Increased phosphorylation of T correlates with complex formation between T and p53 in both SV40-infected and SV40-transformed cells (10, 14) and with the transformed phenotype (14, 41). The phosphorylation state of T also correlates with modulation of a number of replication activities of T and its conformation (34, 41, 49). Differential phosphorylation of several N-terminal sites in T correlates with specific binding to SV40 *ori* DNA sequences and DNA unwinding (23, 44, 45). Cell cycle-dependent specific phosphorylation events can also affect the activities of T; for example, modification of Thr-124 by the cell cycle factor p34-*cdc2* kinase stimulates SV40 DNA replication (27). Individual sites in T have characteristically different turnover rates; phosphothreonine (P-Thr) sites, in general, have greater stability than most phosphoserine (P-Ser) sites (40, 54). Genetic analysis mea-

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suring the effect of mutating individual phosphorylation sites in SV40 T have confirmed a relationship between phosphorylation and both replication and transformation functions (41, 46). However, defining the relationship between T functions, specific phosphorylation, and complex formation with p53 has been more difficult. Mutant 5080 T, defective for p53 binding, both has an altered host range for transformation and is inactive for SV40 DNA replication-related activities (33). While 5080 T is underphosphorylated at both N- and C-terminal sites, it is defective for ATPase and oligomerization activities and is unstable (49).

Changes in the phosphorylation state of p53 have been correlated with modulation of a number of its functions such as cellular transformation and cell cycle regulation (28, 30, 31, 37, 38, 43, 48, 50). The N-terminal phosphorylation sites in murine p53 are located in the same region as the transactivation domain and adjacent to the T-binding domain, while one of the C-terminal sites is adjacent to the nuclear transport sequences (1, 11, 35). However, the role of phosphorylation in regulating the DNA binding, transactivation, and oligomerization activities of p53 and its complex formation with SV40 T is unknown. Furthermore, the phosphorylation sites in monkey p53 from CV1 cells, the normal permissive host for SV40 productive infection, have not been identified.

In this report, we identify the specific in vivo phosphorylation sites in monkey p53 from CV1 cells. We also compared changes in the phosphorylation of free and complexed forms of monkey p53 and SV40 T isolated from uninfected and SV40-infected CV1 cells. We find enhanced phosphorylation of specific N- and C-terminal sites in complexed T relative to free T in productively infected CV1 cells. Furthermore, we find five- to sevenfold increase in the amount of labeled phosphate incorporated into N-terminal phosphoserine sites located between residues 9 and 99 and in Ser-315 for complexed forms of p53 (p53+) relative to free p53 (p53o). We conclude that the enhanced phosphorylation of both T and p53 correlates with the ability of T to form complexes with p53 in SV40-infected and -transformed cells.

(These results were previously reported in preliminary form [L. Tack, J. Wright, A. Srinivasan, J. Pipas, S. Deb, and P. Tegtmeyer, 17th Ann. UCLA Symp. Mol. Cell. Biol., Abstr. O397, p. 158, 4–16 April 1988].)

MATERIALS AND METHODS

Cells, virus, and metabolic labeling. Monkey CV1 cells were grown in Dulbecco modified Eagle medium (DME) plus 5% fetal bovine serum. CV1 cells (15-cm dishes) were infected with SV40 wild-type 800 virus as previously described (52). Each dish, containing 5×10^7 cells, was radiolabeled in vivo with 1 mCi of [^{35}S]methionine (1,250 Ci/mmol; Amersham) for 16 h in 10 ml of DME plus 0.2 N methionine and 5% dialyzed calf serum. For in vivo phosphorylation studies, each dish was labeled with up to 10 mCi of $^{32}P_i$ in 10 ml of phosphate-free DME plus 5% dialyzed calf serum for 4 h. SV40-infected cells were also labeled with 3.3 mCi of [^{35}S]cysteine (1,065 Ci/mmol; Amersham) per dish for 4 h in cysteine-free DME with no serum. Cell extracts were prepared with hypotonic buffer as previously described (52) except that potassium phosphate (10 mM) and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM) and aprotinin (Sigma) were added according to the manufacturers' directions. Lysates were centrifuged at 20,000 × *g* for 45 min to remove cell debris. About 600 μl of extract was recovered per 15-cm dish.

Immunoprecipitation. Extracts of SV40-infected CV1 cells were immunoprecipitated with saturating amounts of either the T-specific antibody PAb 108 (17) or the p53-specific antibody PAb 122 (19) and protein A-Sepharose (Pharmacia) as previously described (52). Sequential immunosorption using PAb 122 followed by PAb 108 was used to generate T+p53 and T without p53 (To) fractions, respectively (51). Extracts of uninfected CV1 cells were reacted with PAb 122 to prepare free p53. The immune complexes were washed three times with NET–Nonidet P-40 buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl [pH 8.0], 0.05% Nonidet P-40, 10 mM phosphate). Immune-complexed T and p53 proteins were analyzed by sodium dodecyl sulfate (SDS)-gel electrophoresis, Coomassie blue staining, and autoradiography. Labeled T and p53 bands were quantified by using an AMBIS beta scanning system (52).

Phosphopeptide and phosphoamino acid analysis. Extracts of one 15-cm plate of SV40 infected cells or three plates of uninfected CV1 cells (each labeled for 4 h with 10 mCi of $^{32}P_i$) were immunoprecipitated as described above. To reduce the background, the final wash of the immune complexes contained 0.5 M NaCl. Each sample was fractionated by SDS-gel electrophoresis, and the wet polyacrylamide gel was exposed for 10 to 30 min at 25°C or for 60 min at −70°C with an intensifying screen. ^{32}P-labeled T and p53 bands, each representing 1×10^7 to 2×10^7 cells, were extracted from wet gels, oxidized, and digested with trypsin as previously described (3) except that trypsin was added at 2% (wt/wt) of sample (including carrier protein) for 30 min at 37°C for a total of four times. This modified protocol, developed originally to prepare tryptic peptides from modified histones, more efficiently digests consecutive arginyl and lysyl residues and reduces the number of contaminating chymotryptic cleavage reactions (48a). ^{35}S-labeled p53 bands were similarly extracted from SDS-polyacrylamide gels and digested with trypsin. Peptides from labeled T were separated on 100-μm cellulose thin-layer chromatographpy (TLC) plates plates by electrophoresis (pH 1.9; 1 kV for 40 min) in the first dimension and chromatography (isobutyric acid-pyridine-acetic acid-butanol-H_2O [65:5:3:2:29 by volume]) in the second (42). Peptides from labeled p53 were separated by electrophoresis at pH 1.9 and then subjected to chromatography (pyridine-acetic acid-butanol-H_2O [50:15:75:60 by volume]) in the second dimension (20, 28). ^{32}P-labeled peptides were visualized by autoradiography with presensitized Kodak X-Omat AR film with intensifying screens at −70°C. ^{35}S-labeled peptides (20,000 cpm per plate) were visualized after spraying with En^{3}Hance and exposing the presensitized film for 7 days at −70°C. The relative amounts of labeled phosphopeptides on autoradiograms exposed for different times were quantified in the linear range, using an LKB UltroScan XL laser densitometer and computer system. Phosphoamino acid analyses were performed by partially hydrolyzing protein or peptide samples in 5.7 M HCl at 110°C for 1 h. The hydrolysates were separated on 100-μm cellulose TLC plates by electrophoresis at pH 1.9 (1.5 kV for 20 min) in the first dimension and pH 3.5 (1.3 kV for 16 min) in the second (7) and visualized by autoradiography.

Secondary digestion (chymotrypsin and V8) of ^{32}P-labeled tryptic peptides was carried out after two-dimensional fractionation as described previously (28). Automated Edman degradation of ^{32}P-labeled tryptic peptides from p53 and T was carried out by Brian Tack (Scripps Research Institute), using a Beckman 890D spinning cup sequencer in the presence of added myoglobin carrier (0.5 nmol) and a Polybrene prerun. Sequence analysis (a minimum of 1,000 cpm per

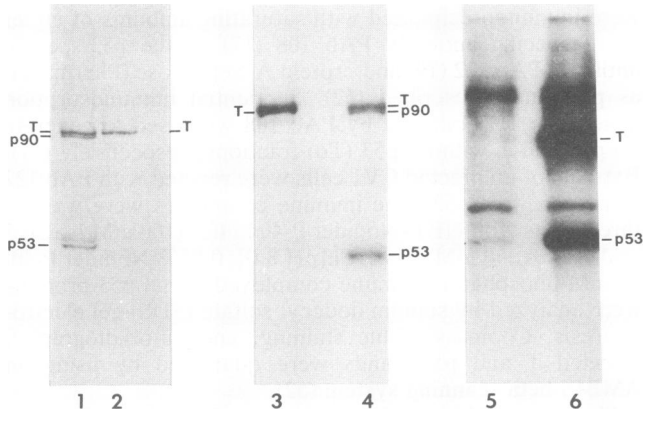

FIG. 1. SDS-gel analysis of free and bound forms of SV40 T and monkey p53. SV40-infected CV1 cells were labeled either for 16 h with 250 μCi of [^{35}S]methionine (lanes 1 and 2) or for 4 h with 5 mCi of $^{32}P_i$ (lanes 3, 4, and 6). Extracts were sequentially immunoprecipitated with PAb 122 to prepare T+p53 complexes (lanes 1, 4, and 6) and PAb 108 to prepare To fractions (lanes 2 and 3). Only 1/10 of the total To sample was loaded in lane 2 to better show the position of the single T band relative to the T+ doublet in lane 1. Uninfected cells were labeled for 4 h with 11 mCi of $^{32}P_i$ and immunoprecipitated with PAb 122 to prepare free p53o (lane 5). Autoradiograms were exposed for 60 min at 25°C (lanes 1 and 2) or for 30 to 60 min at −70°C with an intensifying screen (lanes 3 to 6).

peptide) was carried out for 15 to 20 steps, collecting fractions after each cycle. Initial yields were 50% for the first cycle, with an average consecutive yield of 95% for subsequent cycles. Fractions were dried and solubilized in 10 μl of pH 1.9 buffer, spotted on a cellulose plate, and electrophoresed at pH 1.9 (1 kV for 20 min) along with labeled P_i standards in one dimension. Autoradiography was carried out for 16 days, using presensitized film and an intensifying screen as before.

RESULTS

Analysis of T and p53: T bound to p53 is more highly phosphorylated than free T. SV40-infected and uninfected CV1 cells were labeled in vivo for 4 h with $^{32}P_i$ or with [^{35}S]methionine for 16 h as described in Materials and Methods. Hypotonic extracts were sequentially immunoprecipitated with the p53-specific monoclonal antibody PAb 122, coprecipitating p53 in complex with T (T+p53 fraction), and then with the SV40 T-specific monoclonal antibody PAb 108, precipitating T depleted in p53 (To fraction). Free monkey p53 (p53o) from uninfected CV1 cell extracts was immunoprecipitated with PAb 122. SDS-gel analysis of the T+p53, To, and p53o fractions is shown in Fig. 1. For ^{35}S- and ^{32}P-labeled T+p53 (lanes 1 and 3, respectively), there were three major bands: a doublet of 94-kDa (94K) and 90K bands and the p53 band. For ^{35}S- and ^{32}P-labeled To fractions (lanes 2 and 4, respectively), only the upper 94K band was present, as previously reported (45, 49). ^{32}P-labeled free p53 is shown in lane 5.

Only 6% of ^{35}S-labeled total T is bound to p53, while greater than 95% of p53 is bound to T in SV40-infected CV1 cells, as previously shown (18, 51). About 15 to 20% of ^{32}P-labeled T is bound to p53. Quantification of the ^{32}P- and ^{35}S-labeled bands from eight different preparations indicated that the specific activity (ratio of ^{32}P to ^{35}S label) of T+ was consistently 2.6-fold greater than that of To, on average. Similar ratios were obtained when T was quantitated by Coomassie blue staining instead of metabolic labeling with methionine. An increase in labeled T+ relative to To was also observed for SV40-infected cells labeled with ^{32}P for 12 h. In contrast, comparison of the phosphorylation levels in bound and free p53 proteins was difficult because of their very disparate half-lives. Monkey p53o from normal CV1 cells, like free mouse p53 (18), is unstable ($t_{1/2}$ < 30 min) compared with p53+ complexed with SV40 T ($t_{1/2}$ > 24 h).

We have previously reported the presence of a p90 protein in the T+p53 complex that is absent in T fractions lacking bound p53 isolated from lytically infected CV1 cells (51). A similar T+p53+p90 complex was isolated from wild-type SV40-transformed C3H10T1/2 (SV10T) cells but was absent in mutant 5080-transformed 10T1/2 (5080-10T) cells which express mutant T defective for p53 binding (49). p90 may be a cellular protein or an altered form of T (see below).

Tryptic phosphopeptide analysis of To and T+ antigen. To analyze the ^{32}P-labeled phosphotryptic peptide pattern of the T+ and To fractions, ^{32}P-labeled proteins were extracted from SDS-gels, oxidized, and digested with trypsin, and the phosphopeptides were separated in two dimensions as described in Materials and Methods (Fig. 2). Peptides were numbered according to Scheidtmann et al. (41, 42). Six known phosphorylated tryptic peptides have been identified in total T; peptide 1 is phosphorylated at Ser-639, peptides 2 and 4 are partially digested tryptic peptides both phosphorylated at Ser-639, peptide 3 contains one or more residues phosphorylated at Ser-106, Ser-112, Ser-120, Ser-123, and Thr-124, peptide 5 is phosphorylated predominantly at Ser-677 and Ser-679, and peptide 6 is phosphorylated at Thr-701.

When equal amounts of ^{32}P-labeled tryptic digests of T+ and To were compared under identical conditions, several differences were consistently apparent. Tryptic digestion of To, representing 90% of total T and 80% of ^{32}P-labeled T, generated six major phosphopeptides similar to those reported for total unfractionated T (42). For T+, the same six phosphopeptides were present, and a mix confirmed that they were identical to those present in To (data not shown). Peptides 3 and 5 were significantly more labeled in T+ than in To. In addition, peptide 5 (containing serines 657 to 679) had multiple spots, 5a to 5d, appearing on a diagonal, consistent with the presence of mono-, di-, tri-, and tetraphosphorylated forms which have incrementally increased negative charge and polarity (resulting in reduced vertical mobility in the hydrophobic chromatography buffer). Only the monophosphorylated peptide, 5a was present in To. Peptide 3 migrated as a smear, as expected for its 60-amino-acid size, heterogeneous basic C terminus, and multiple phosphorylation sites (42).

The lytic T+ fraction contained several new labeled tryptic peptides, X, Y, and Z. The most prominant, X, was extremely hydrophobic. These peptides were absent from digests of To (representing > 90% of T), which contains only the 94K band, and were not previously observed for total lytic SV40 T samples (42). Peptides X, Y, and Z appear to correlate with the presence of p90 in T+p53 complexes (Fig. 1) (49, 51). The To fraction consistently contained peptide A, which was not present in the T+ fraction.

Edman sequencing of peptide 6 yielded $^{32}P_i$ at position 4, as expected for a tryptic peptide containing Thr-701. Sequence analysis of peptide X yielded $^{32}P_i$ at position 10. Both peptides 6 and X were resistant to V8 but were sensitive to chymotrypsin digestion. Two-dimensional tryptic peptide analysis of T+ and To samples labeled in vivo with [^{35}S]Cys suggested that peptide X contained cysteine. This was

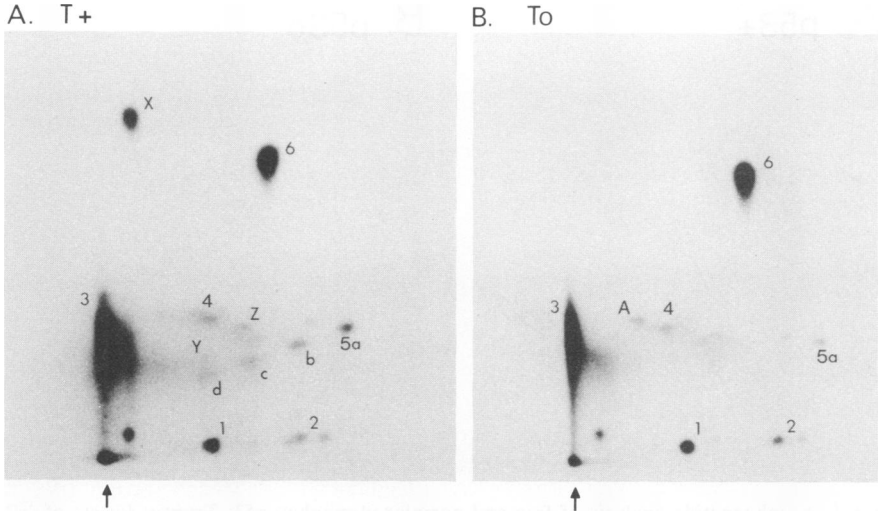

FIG. 2. Two-dimensional phosphopeptide analysis of free and complexed SV40 T. Tryptic digests of ^{32}P-labeled T+ and To bands excised from SDS-gels (lanes 3 and 4 in Fig. 1) were each separated on TLC plates by electrophoresis at pH 1.9 (horizontal direction) and chromatography (vertical direction) as described in the text. The origin is indicated by a vertical arrow. Equal counts (5,000 Cerenkov cpm) were loaded on each plate. Autoradiograms were exposed for 22 h at −70°C with an intensifying screen.

confirmed by elution, mixing, and the observed comigration of ^{32}P- and ^{35}S-labeled peptides when analyzed by two-dimensional mapping. The net neutral charge/mass ratio at pH 1.9 was consistent with the presence of two negatively charged residues such as cysteic acid and P-Thr. Upon inspection of all threonine-containing tryptic peptides in T, peptide 14 (residues 155 to 165, located in the DNA binding domain of T) was the best candidate for X. However, assessment of the net charge/mass ratio observed for chymotrypsin-digested X (+0.09) and calculated for chymotrypsin-digested peptide 14 (+0.33) in T appeared to rule out Thr-164 in this peptide as the source of phosphopeptide X. Further evidence of the nature of p90 comes from two-dimensional tryptic peptide analysis of a ^{32}P-labeled 90K band complexed with free p53 (precipitated by anti-p53 but not by anti-T) in 5080-10T cells (data not shown). This polypeptide, presumed to be a mouse form of p90, generated four major phosphopeptide spots whose mobilities corresponded to the X, Y, and Z spots present in the T+ sample from infected CV1 cells (Fig. 2) and in total T from SV-10T cells (49). These data suggest that p90 is either an unusually phosphorylated distinct form of T or a new cellular phosphoprotein that complexes with p53.

Phosphoamino acid analysis. Phosphoamino acid analysis was performed on ^{32}P-labeled T+, To, p53+, and p53o proteins and their isolated phosphopeptides. For T+ samples, the ratio of P-Ser to P-Thr (3.7) was slightly higher than for To samples (3.3). The phosphoamino acid content of several labeled T peptides isolated after fractionation in two dimensions is shown in Fig. 3. Peptide X contained P-Thr, peptide Y contained P-Ser, and peptide Z contained P-Ser and a small amount of P-Thr. Peptides 1, 2, 4, and 5a to 5d all contained P-Ser, peptide 6 contained P-Thr, and peptide 3 contained both P-Ser and P-Thr (not shown), in agreement with previous reports (42). For To, peptide A contained P-Ser. Phosphoamino acid analysis of free and bound forms of monkey p53 indicated that P-Ser was the only phosphorylated amino acid present (not shown), similar to results for mouse p53 (28). Peptides M1 and M2, individually eluted from tryptic maps of p53+, contained P-Ser, as expected.

Tryptic phosphopeptide analysis of free and complexed monkey p53. Free and bound forms of p53 labeled in vivo with ^{35}P were isolated from SV40-infected and uninfected CV1 cells by using monoclonal anti-p53 as described in Materials and Methods. The phosphorylation patterns of both forms of monkey p53 were analyzed by two-dimensional tryptic peptide mapping (Fig. 4). p53+ yielded four major phosphopeptides, M1 to M4, and two minor phosphopeptides, X and Y, on the same diagonal as M3 (presumed to be multiphosphorylated forms of M3). Phosphopeptide F appeared to be a minor multiphosphorylated form of M4. A qualitatively similar pattern of labeled pep-

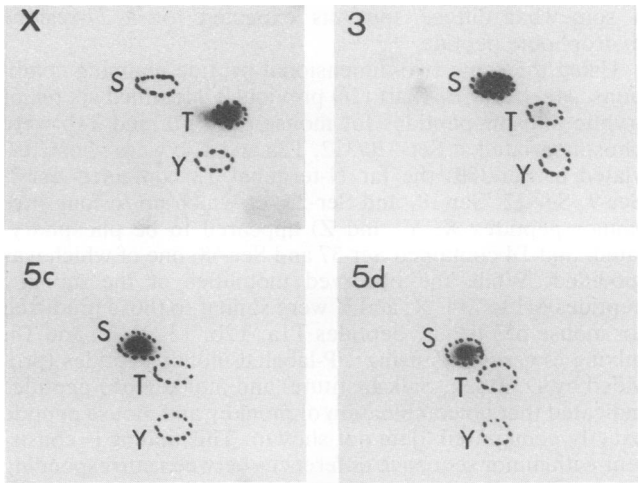

FIG. 3. Phosphoamino acid analysis. Tryptic phosphopeptides derived from complexed T+ were isolated from the TLC plates shown in Fig. 2, hydrolyzed, and analyzed by electrophoresis in two dimensions as described in the text. The positions of stained, unlabeled phosphotyrosine (Y), phosphoserine (S), and phosphothreonine (T) are indicated by dotted lines. Autoradiograms were exposed for 5 days at −70°C with an intensifying screen.

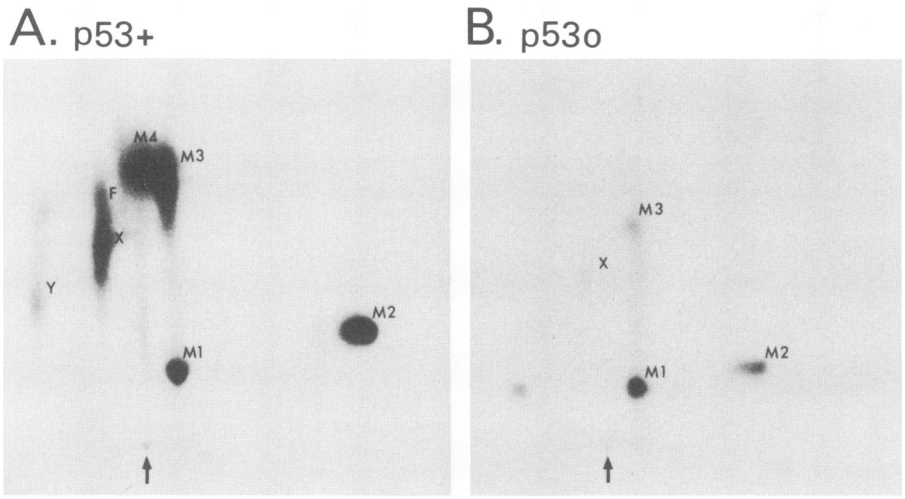

FIG. 4. Two-dimensional phosphopeptide analysis of free and complexed monkey p53. Tryptic digests of ^{32}P-labeled p53+ and p53o excised from SDS-gels (lanes 5 and 6 in Fig. 1) were separated at pH 1.9 (horizontal direction) and chromatography (vertical direction) as described in the text. About 1,000 and 500 Cerenkov cpm were loaded on the p53+ and p53o plates, respectively. Autoradiograms were exposed for 2 days at −70°C with an intensifying screen.

tides was observed when monkey p53o was mapped. However, the relative intensities of M3, X, and M2 were significantly reduced, and M4, Y, and F were barely detectable. The same pattern of phosphopeptides was obtained for p53+ precipitated from SV40-infected cells with anti-p53 or anti-T PAb 108 (data not shown).

Identification of phosphorylation sites in monkey p53. Using the known amino acid sequence of monkey p53 (27), we calculated and plotted the predicted two-dimensional mobilities of all serine-containing tryptic peptides (data not shown). The observed mobilities of the ^{32}P-labeled monkey phosphopeptides M1, M2, M3, X, Y, F, and M4 shown in Fig. 4 corresponded best to the predicted peptides containing residues 1 to 24 (M3), di- and triphosphorylated M3 (X and Y), 25 to 101 (M4), diphosphorylated M4 (F), 307 to 320 (M2), and 386 to 393 (M1). Monkey peptide M4 migrated as a somewhat diffuse spot, as expected for a 77-residue hydrophobic peptide.

Using the same two-dimensional peptide mapping conditions, Meek and Eckhart (28) previously identified six major tryptic phosphopeptides for mouse p53; T1a and T1b were phosphorylated at Ser-310/312, T2a and T2b were phosphorylated at Ser-389, the far N-terminal T3 contained Ser-7, Ser-9, Ser-12, Ser-18, and Ser-23, of which up to four sites (minor peptides X, Y, and Z) appeared to be phosphorylated, and T4 contained Ser-37 and Ser-58, one of which was modified. While the observed mobilities of the monkey peptides M1 to M4, X, and Y were similar to those predicted for mouse p53 tryptic peptides T1a, T2b, T3, X, Y, and T4, mixing experiments using ^{32}P-labeled mouse peptides (provided by D. Meek, Salk Institute) and monkey p53 peptides indicated that no combination of monkey and mouse peptide exactly comigrated (data not shown). This finding is consistent with minor sequence differences between corresponding mouse and monkey tryptic peptides. No monkey phosphopeptides corresponded to mouse peptides T1b and T2a. This can be explained by comparing monkey and mouse p53 sequences and noting the absence of consecutive lysines in monkey peptides M1 and M2, a proline adjacent to lysine (trypsin-resistant) in monkey M2, and the known preference of trypsin for arginyl rather than lysyl residues (affecting monkey but not mouse M2). In addition, our modified trypsin digestion protocol utilizes four consecutive additions of small amounts of enzyme for short times (see Materials and Methods). This minimizes incomplete digestion of peptides, in particular those containing multiple adjacent basic residues, and reduces chymotrypsin contamination. With long digestion times, trypsin is inactivated due to autodigestion, resulting in a lower yield of the less cleavable peptide bonds in the target molecule.

Secondary digestion results indicated that monkey peptides M3, M4, and X were sensitive to V8 and chymotrypsin whereas M2 was resistant to both enzymes (data not shown). Chymotrypsin digestion of M3 yielded a major spot that was more basic and hydrophobic than M3 and two minor spots. Inspection of the sequence of tryptic peptide 1–24 indicated that Ser-20 is most likely the major ^{32}P-labeled site in M3. Surprisingly, chymotrypsin digestion of X (presumed to be diphosphorylated M3) yielded a major peptide with a net acidic charge and a more polar composition. This finding suggests that X contains two phosphorylated residues at Ser-9 and Ser-15 which are N terminal to Phe at position 19. M4 gave two major labeled peptides after both V8 and chymotrypsin digestion, suggesting the presence of two phosphorylation sites. The new mobilities were consistent with at least one site being located at serines 90 to 99, near the carboxy terminus of peptide 25–101. The results of V8 digestion indicated that the other site in M4 is located at Ser-33 or Ser-37. Monkey M4 was more negatively charged than mouse T4 in peptide mixing experiments. Peptide F is likely diphosphorylated M4. We conclude that M4 contains two phosphorylation sites in p53+, with at least one of these sites located at serines 90 to 99.

Automatic Edman sequential degradation of peptide M2 released a major peak of $^{32}P_i$ at cycle 9, corresponding to phosphorylation of Ser-315 and not Ser-313 or Ser-314. Surprisingly, sequence analysis of M1 containing Ser-392 failed to yield detectable labeled P_i in any fraction despite sequencing 4,000 cpm and the short seven-residue length of this peptide. Ser-389 in mouse p53 is reported to be covalently bound to RNA via a 5′ linkage (39). If Ser-392 was similarly modified in monkey p53, it may not undergo beta

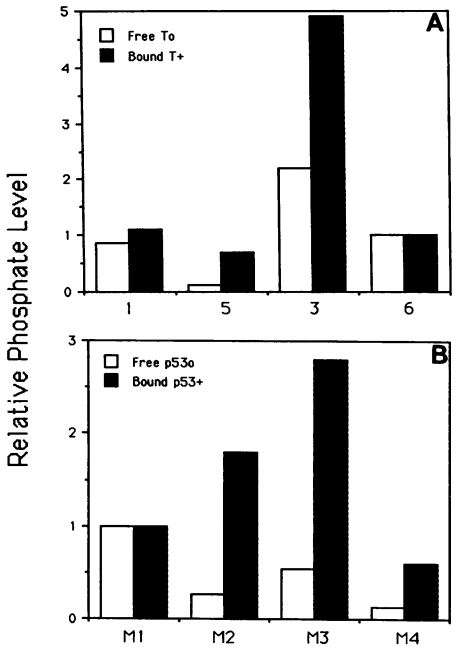

FIG. 5. Relative amounts of ^{32}P-labeled phosphopeptides in bound compared with free SV40 T and monkey p53. Quantification was done as described in the text. For T (A), the ^{32}P label was normalized (set to 1.0 mol of phosphate per mol of peptide) to peptide 6 containing Thr-701. Peptide 1 contains Ser-639 (and includes labeled peptides 2 and 4), peptide 5 contains serines 657 to 679, and peptide 3 contains serines 106 to 123 and Thr-124. For monkey p53 (B), the ^{32}P-labeled peptide M1 (containing Ser-392) was set equal to 1.0 mol of phosphate per mol of peptide. Peptide M2 contains Ser-315, peptide M3 (plus X and Y) contains Ser-9, Ser-15, and Ser-20, and peptide M4 is modified at one of serines 90 to 99 and at Ser-33 or Ser-37.

elimination and liberate free P_i upon Edman degradation as do normal phosphoserine monoesters. M3 gave a faint labeled spot at position 9 (corresponding to Ser-9) after Edman degradation; no label was released at position 6. Two-dimensional mapping of monkey p53 labeled in vivo with [^{35}S]cysteine indicated the presence of a Cys-containing peptide with the same mobility as phosphorylated M2 (data not shown). However, monkey M2 should not contain cysteine. Further inspection of the monkey p53 sequence indicated that one Cys-containing peptide (residues 182 to 196) is predicted to have the same mobility as phosphorylated M2.

In summary, we have identified the monkey p53 phosphotryptic peptides and their corresponding phosphoserine residues. They are M1 (TEGPDSD) modified at Ser-392, M2 (ALPNNTSSSPQPKK) modified at Ser-315, M3 (MEEPQS DPSIEPPLSQETFSDLWK) modified primarily at Ser-20, X (diphosphorylated M3) modified at Ser-9 and Ser-15, and M4 (LLPENNVLSPLPSQAVDDLMLSPDDLAQWLTEDPG PDEAPRMSEAAPHVAPTPAAPTPAAPAPAPSWPLSSS VPSQK) modified at two sites, including one or more of serines 90, 94 to 96, or 99 and either Ser-33 or Ser-37.

Quantification of specific phosphorylation sites in free and bound forms of T and p53. We measured the relative amounts of ^{32}P-labeled peptides 1 to 6 in free and complexed T by densitometric analyses of the autoradiograms. The data (Fig. 5A) are averages of four experiments. The phosphate content for peptide 6 containing Thr-701 was set equal to 1.0, as this site in T appears to be stoichiometrically and stably modified shortly after synthesis (40, 42). For T+, the relative amount of phosphate at Ser-639 (predominantly peptide 1 but also peptides 2 and 4) was 1.1 ± 0.2 mol, the value for peptide 5a was 0.7 ± 0.2 mol, and the value for peptides 3 and 3a was 4.9 ± 0.8 mol (suggesting that Ser-106, Ser-112, Ser-120, Ser-123, and Thr-124 are fully modified). The amounts of labeled phosphate in peptides 5a to 5d (assumed to be related mono-, di-, and triphosphorylated forms and containing serines 657 to 679) were combined. Overall, there was 7.7 ± 0.8 mol of labeled phosphate per T+ molecule. Peptide X, containing 0.7 ± 0.2 mol of labeled phosphate, was not included. For To, when the amount of labeled phosphate in peptide 6 was made equal to 1.0, the value for peptides 1, 2, and 4 was 0.9 ± 0.1 mol, the value for peptide 5 was 0.1 ± 0.05 mol (with only monophosphorylated 5a present), and the value for peptide 3 was 2.2 mol. A total of 4.2 ± 1 mol of labeled phosphate was calculated for To.

The change on comparing bound and free forms of T was a 1.3-fold increase for peptide 1 containing Ser-639, a 6.4-fold increase for peptide 5 containing serines 657 to 679, and a 2.2-fold increase for peptide 3 containing residues 105 to 124. Both the amount of label and the number of multiphosphorylated forms of peptide 5 increased in T+ compared with To. The P-Ser/P-Thr ratio for peptide 3 increased for T+ compared with To. Overall, there was a net increase of 3.5 mol of phosphate in T+ compared with To.

The relative amounts of ^{32}P-labeled peptides M1 to M4 in free p53o and complexed p53+ proteins were measured by densitometric analyses of the autoradiograms. The data (Fig. 5B) are averages of four experiments. For each sample, the most labeled peptide was made equal to 1.0 mol of labeled phosphate and normalized to the values of the other peptides. For p53+, the most labeled peptide was M2 (Ser-315). M3 (labeled at up to three different serine residues and including X and Y) was then estimated to have a total phosphate content of 1.56 ± 0.15 mol, the content for M1 was 0.56 ± 0.09 mol, and the content for M4 was 0.33 ± 0.05 mol distributed between two sites. For p53o, M1 (Ser-392) was the most labeled peptide. Almost no detectable labeled multiphosphorylated peptide M3 (X or Y) or M4 was observed. When M1 in p53o was made equal to 1.0, the relative labeled phosphate contents were estimated to be 0.26 + 0.20 mol in M2, 0.54 + 0.26 mol in M3, and 0.13 + 0.08 mol in M4. When M1 in both p53o and p53+ was adjusted to the same relative value (1.0), then M2 was 7-fold, M3 was 5.2-fold, and M4 was 4.6-fold more modified for p53+ than for p53o (Fig. 5). Regardless of whether we normalize the labeled phosphoserine sites to Ser-315 or to Ser-389, the result is the same: increased phosphorylation of peptides M2, M3 (plus X and Y), and M4 in complexed p53+ compared with free p53o. We estimate there are 3.2 ± 0.1 mol of phosphate per molecule of p53+. The extent of p53 labeling is assumed to reflect the extent of phosphorylation at a particular site. However, these values may be affected by differential turnover of individual phosphate sites; for example, stable sites may not turn over in a 4-h labeling period and would then be underrepresented. The phosphorylation content of the free p53o protein was not estimated because of its short half-life.

DISCUSSION

We have identified the in vivo phosphorylation sites in monkey p53 from SV40 lytically infected and uninfected CV1 cells. In addition, we compared the changes in the

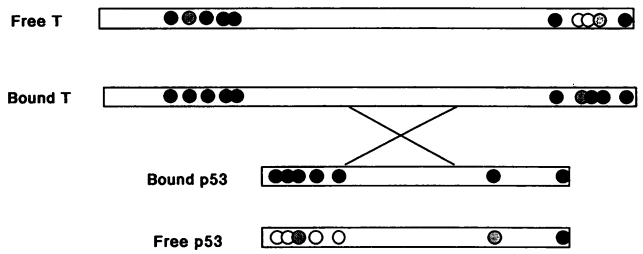

FIG. 6. Summary: specific phosphorylation sites in free and complexed forms of SV40 T and monkey p53 in lytically infected cells. Increasing phosphorylation is shown by increasing shading of circles, with solid circles indicating 100% modification and open circles less than 5% modification. The phosphorylation sites shown for T are Ser-106, Ser-112, Ser-120, Ser-123, and Thr-124 located at its N terminus and Ser-639, one or more of Ser-657 to Ser-679, and Thr-701 at its C terminus. The phosphorylation sites for monkey p53 are Ser-9, Ser-15, Ser-20, Ser-33, or Ser-37, at least one of serines 90 to 99, Ser-315, and Ser-392. For T, Thr-701 is shown modified at 1 mol of phosphate per molecule (see text). For both forms of p53, the relative amount of labeled phosphate was made relative to Ser-392, with Ser-315 in p53+ shown as 100% modified. The extent of labeling was assumed to reflect the actual extent of phosphorylation at a particular site (see text).

phosphorylation state of free and complexed forms of SV40 T and monkey p53. T complexed with p53 and free T fractions were isolated from extracts of SV40-infected CV1 cells metabolically labeled with $^{32}P_i$ by sequential immunoprecipitation with monoclonal anti-p53 and anti-T. Free p53 was isolated from uninfected CV1 cell extracts by using anti-p53. Tryptic phosphopeptides were separated in two dimensions and then further analyzed and quantified. The data shown in Fig. 5 are diagrammed in Fig. 6. Our results indicate that there is enhanced phosphorylation of several sites in complexed forms of both T and monkey p53 from infected CV1 cells.

The in vivo phosphorylation sites in monkey p53 isolated from CV1 cells were localized to both N- and C-terminal sites and include Ser-9, Ser-15, Ser-20, Ser-315, Ser-392, and two or more serines located between Ser-33 and Ser-99. When complexed monkey p53 from SV40-infected CV1 cells was compared with free p53 from uninfected CV1 cells, we observed a fivefold increase in specific phosphorylation of the N-terminal peptides M3 (including Ser-9, Ser-15, and Ser-20) and M4 (containing at least two phosphoserine sites) and a sevenfold increase in the C-terminal peptide M2 (Ser-315). These results are qualitatively similar to those of Samad et al. (38), who identified phosphorylation sites within the first N-terminal 98 residues and at Ser-312 and Ser-389 in mouse p53 isolated from SV40-transformed cells.

On comparing the phosphorylation patterns of complexed and free forms of mouse p53 isolated from SV40-transformed and normal C3H10T1/2 (50) cells, we found differences similar to those observed here for analogous forms of monkey p53 from SV40-infected and normal CV1 cells. Meek and Eckhart (28) found just a twofold increase in Ser-312 phosphorylation for mouse p53+ compared with p53o from SV40-transformed and normal 3T3 cells. However, we have observed that ^{32}P labeling for 12 h or more of either SV40-infected CV1 cells or SV40-transformed C3H10T1/2 cells resulted in a significant decrease in phosphorylation of peptides M3, M4, and M2 in p53+ relative to p53o (50).

The transactivation domain in p53 (35) is located in the same region as the N-terminal phosphorylation sites, suggesting that the phosphorylation state of this region may regulate its function in activating target promoter sequences.

Ser-312/315 in p53 is the primary site of phosphorylation when either mouse or monkey p53 is complexed with T in both infected and transformed cells (21; this report). This site is a substrate for the cell cycle-regulated p34-*cdc2* kinase (4). Thus, cell cycle-dependent modification of Ser-312 in mouse p53 or Ser-315 in monkey and human p53 is expected to affect its antiproliferative activity. Surprisingly, mutation of Ser-312 in mouse p53 had no detectable effect on its complex formation with SV40 T or its reactivity with PAb 246, a p53-specific conformation-dependent antibody (29). These results suggest that Ser-312 does not significantly affect the normal wild-type structure of p53 in its T-binding domain.

Ser-389 in mouse p53 has been shown to be an in vitro substrate for casein kinase II (23). However, Ser-389 in mouse p53 is covalently attached to RNA in vivo (39). A similar modification of a C-terminal serine residue is also present in SV40 T (5). Decreased phosphorylation of Ser-389 in mouse p53 in SV40 *tsA*58-transformed mouse cells was observed during temperature shift experiments (38). This may be a consequence of temperature effects on activation of different cellular kinases and phosphatases rather than a failure to form the T+p53 complex at the nonpermissive temperature.

Patschinsky and Deppert (32) found little difference in the phosphorylation state of p53 in normal compared with SV40-transformed BALB/c 3T3 cells, although they compared dissimilar labeling times (2 h versus 17 h, respectively) and used different cell types. Since the magnitude of the specific changes in p53 is affected by metabolic labeling time and suggests a differential turnover rate for the individual sites, variations in labeling time, temperature, and other growth-related factors are likely to alter the activity of various modifying enzymes.

For complexed T+ compared with free To, there was increased phosphorylation of both N- and C-terminal serines. Phosphorylation sites located in the tryptic peptide containing residues 106 to 124 were about twofold more labeled and appeared to be fully modified in T+. The tryptic peptide containing serine residues 657 to 679 was about sixfold more labeled. Ser-639 and Thr-701 appeared unchanged. A total increase of 3.5 mol of labeled phosphate was calculated for T+/To. New phosphotryptic peptides X (containing P-Thr), Y, and Z (both containing P-Ser) were also present in the T+ sample and correlated with the p90 protein present in T+p53 complexes. It is likely that additional sites besides Ser-677 and Ser-679 are modified in peptides 5a to 5d from bound T+ shown in Fig. 2. Ser-665 and Ser-667 are phosphorylated in SV40 T purified from baculovirus vector-infected insect cells (6).

The phosphopeptide pattern for SV40 T+ (bound to monkey p53) in lytically infected CV1 cells was essentially identical to that of wild-type SV40 T complexed with p53 expressed in SV-10T cells mapped under identical conditions (49). Furthermore, the phosphopeptide pattern for lytic To (not bound to p53) was the same as for mutant 5080 T (defective for p53 binding) isolated from 5080-10T cells (49). Thus, altered phosphorylation of T+/To corresponds with complex formation and does not appear related to species-specific expression in monkey compared with mouse cells or in SV40 lytically infected compared with transformed cells. Furthermore, altered phosphorylation of T does not seem to correlate with T stability, since lytic To and T+ have similar half-lives (48a).

The relationship between altered phosphorylation of SV40 T complexed with p53 and the activities of T that correlate with complex formation is unclear. Mutation of individual phosphorylation sites in T alters both its replication and transformation functions; the N-terminal sites (Ser-120, Ser-123, and Thr-124) correlate with viability, and the C-terminal sites (Ser-677 and Ser-679) correlate with decreased transformation frequency (41, 46). Coincidentally, we find that T+ from either infected or transformed cells exhibits enhanced phosphorylation of these same N- and C-terminal sites (49; this report). Specific phosphorylation of T alters its binding to the SV40 *ori* region (see review in reference 34). Furthermore, complex formation between p53 and T affects a number of T activities, including its binding to specific *ori* DNA sequences (13, 51, 55). Mutant 5080 T, defective in p53 binding and underphosphorylated at N-terminal serines 106 to 123 and at C-terminal serines 657 to 679 (33, 49), has many affected activities (altered host range for transformation, nonviable, ATPase inactive, unstable, and over-expressed compared with wild-type T complexed with p53). This makes it difficult to correlate changes in specific phosphorylation sites with the inability of T and p53 to stably associate. Regardless, these results suggest that some T complexed with p53 may actively participate in both cellular and SV40 replication- and transformation-related activities and not simply represent inactive T or p53 forms. We expect that altered phosphorylation of associated forms of T and p53 will be found to play a significant role in regulating the functions of this and other complexes formed between p53 and viral antigens.

Other factors besides complex formation may affect the phosphorylation state of the T+p53 complex. The kinetics of T and p53 phosphorylation relative to complex formation is unclear. Since many of the phosphorylation sites in T, and probably p53, are metabolically unstable (40), T+p53 complex formation may alter the turnover of these sites. Scheidtmann and Haber (43) suggest that expression of SV40 T induces or activates a protein kinase that modifies p53 in either infected or transformed rat cells. The association of the p90 protein with the T+p53 complex in both SV40-infected and SV40-transformed cells has been previously reported (49, 51). Phosphopeptide analysis of a p90 protein complexed with p53 in mouse C3H10T1/2 cells transformed by mutant 5080 T (defective for p53 binding) indicates that this p90 has a phosphorylation pattern distinct from that of normal T (data not shown). p90 is either an unusually modified form of T or a cellular protein that binds to p53. Further experiments are necessary to characterize this polypeptide.

Together, the results described in this report suggest that increased phosphorylation of both T and p53 is related to the ability of T to form a complex with p53. Specific phosphorylation of T and/or p53 may be either a prerequisite or a consequence of complex formation. Regardless of the temporal relationship between complex formation and enhanced phosphorylation of both proteins, factors such as species and specific cell type, while having some effect, may not be as significant as the direct interaction between T and p53 proteins.

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