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Properties of a Simian Virus 40 Mutant T Antigen Substituted in the Hydrophobic Region: Defective ATPase and Oligomerization Activities and Altered Phosphorylation Accompany an Inability To Complex with Cellular p53

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We have analyzed the biochemical properties of a nonviable simian virus 40 (SV40) mutant encoding a large T antigen (T) bearing an amino acid substitution (Pro-584–Leu) in its hydrophobic region. Mutant 5080 has an altered cell type specificity for transformation (transforming mouse C3H10T1/2 but not rat REF52 cells), is defective for viral DNA replication, and encodes a T that is unable to form a complex with the cellular p53 protein (K. Peden, A. Srinivasan, J. Farber, and J. Pipas, Virology 168:13–21, 1989). In this article, we show that 5080-transformed C3H10T1/2 cell lines express an altered T that is synthesized at a significantly higher rate but with a shorter half-life than normal T from wild-type SV40-transformed cells. 5080 T did not oligomerize beyond 5 to 10S in size compared with normal T, which oligomerized predominantly to 14 to 20S species. In addition, the 5080 T complex had significantly decreased ATPase activity and had a 10-fold-lower level of in vivo phosphorylation compared with that of normal T. Two-dimensional phosphopeptide analysis indicated several changes in the specific ^{32}P labeling pattern, with altered phosphorylation occurring at both termini of the mutant protein compared with the wild-type T. Loss of p53 binding is therefore concomitant with changes in ATPase activity, oligomerization, stability, and in vivo phosphorylation of T and can be correlated with defective replication and restricted transformation functions. That so many biochemical changes are associated with a single substitution in the hydrophobic region of T is consistent with its importance in regulating higher-order structural and functional relationships in SV40 T.

Large T antigen (T), encoded by simian virus 40 (SV40), is required for productive viral infection and for cell transformation. At least three biochemical activities of T are required for SV40 DNA synthesis: *ori*-specific DNA binding, ATPase, and helicase. T undergoes several posttranslational modifications, including oligomerization and phosphorylation (for a recent review, see reference 8). A cellular protein, p53, is found complexed with T in both SV40-infected and transformed cells. p53 has a role in regulating cell cycle processes in normal cells and has oncogenic potential (for recent reviews, see references 7 and 15).

The 5080 series (15a) of SV40 mutant T's has altered sequences near the C-terminal border of the ATPase domain (4) (Fig. 1). This region (between residues 570 to 590) contains the major stretch of hydrophobic amino acids (15a) in SV40 T and is adjacent to the first (Ser-639) of the C-terminal stretch of phosphorylated serine and threonine residues (16). Others (15a) have shown that most mutants in this series are nonviable, are defective for viral DNA replication, and in addition, display a cell type specificity for transformation, in that they are capable of transforming a mouse C3H/10T1/2 cell line but not a rat REF52 cell line. None of the T's from the defective mutants form a complex with p53 (15a). It is possible that other T activities are altered in these mutants to account for their different replication and transformation activities. Therefore, we chose to further study mutant 5080 as representative of this series. Mutant 5080 carries an amino acid substitution of Pro-584–Leu in SV40 T and is defective for both viral DNA replication and transformation of REF52 cells (15a). Amino acid 584 is conserved in five of the six papovavirus T's that have been sequenced; in polyomavirus, the corresponding Pro position is instead encoded as Thr (residue 731).

In this article, we have analyzed a number of properties of 5080 T expressed in transformed mouse C3H10T1/2 cells, including its relative rate of synthesis and stability, ATPase activity, degree of oligomerization, and pattern of in vivo phosphorylation sites. Essentially all of these activities were altered for the mutant, suggesting that the hydrophobic region of T can affect a number of the structural and functional properties of SV40 T.

MATERIALS AND METHODS

Growth of cells and metabolic labeling. Normal mouse C3H10T1/2 cells (obtained from L. Sompayrac, University of Colorado) and mutant 5080-transformed and wild-type SV40-transformed C3H10T1/2 (5080-10T and SV-10T, respectively) cell lines (15a) were grown in Dulbecco modified Eagle medium (DME) plus 10% fetal bovine serum. Cells were radiolabeled for various times for up to 16 h in vivo with 1.0 mCi of [^{35}S]methionine (Amersham Corp.; 1,250 Ci/mmol) per 10-cm-diameter dish in 5 ml of DME plus 0.2 N methionine and 5% dialyzed calf serum. For pulse-chase experiments, cells were labeled for 1 h with [^{35}S]methionine in 3 ml of DME without methionine plus 2% dialyzed calf serum and then chased for 3 h with DME (plus methionine)

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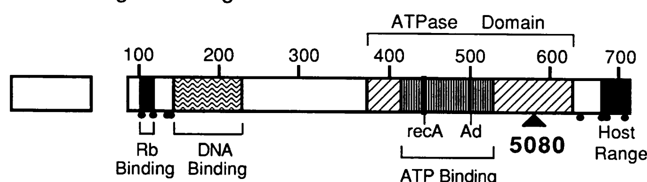

FIG. 1. Map of the primary structure of SV40 T (7a, 8). The approximate locations of the retinoblastoma (Rb)- and DNA-binding sites, the ATPase domain (including the ATP-binding pocket, the *recA*-homologous sequence, and the putative adenylylation (Ad) site), and the host range domain are shown. The location of the 5080 mutation at residue 584 is shown (15a). The locations of phosphorylated residues are indicated (●).

plus 5% fetal calf serum. For in vivo phosphorylation studies, cells were labeled for 4 h with 1 mCi of $^{32}P_i$ in 3 ml of phosphate-free DME plus 5% dialyzed calf serum. Cell extracts were prepared by using a hypotonic buffer as previously described (22) except aprotinin (10 KIU/ml) and potassium phosphate (10 mM) were added. The protease inhibitor aprotinin (Sigma Chemical Co.) was added to the lysis buffer according to the directions of the manufacturer. Lysates were centrifuged at 20,000 × *g* for 45 min to remove cell debris. About 400 μl of extract was recovered per dish.

Immunoprecipitations. Labeled cell extracts were immunoprecipitated with T-specific antibody PAb 419 (12) or PAb 109 (11) and the p53-specific PAb 122 (10) and protein A-Sepharose as previously described (22). NS1 medium was used as a nonspecific control. Sequential immunoprecipitation with anti-p53 followed by anti-T was used to prepare T+p53 and T depleted in p53 fractions (21). Washed immunoprecipitates were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography. Labeled T and p53 bands were quantified by using an AMBIS beta scanning and IBM computer system (23).

ATPase and oligomerization assays. To measure ATPase activity, immune complexes, each derived from 400 μl of extract labeled with [^{35}S]methionine for 16 h in vivo, were washed twice with ATPase buffer (25 mM piperazine-*N*,*N*'-bis(2-ethanesulfonic acid) [PIPES] [pH 7.0], 100 mM NaCl, 5 mM $MgCl_2$, 0.01% Nonidet P-40) and assayed for the rate of conversion of ATP to ADP, using [α-^{32}P]ATP (Dupont NEN Research Products; 750 Ci/mmol) at 33°C (23), with cold ATP added to a final concentration of 2 μM. The immune complexes were then analyzed by SDS gel electrophoresis and autoradiography; ^{35}S-labeled T and p53 bands were quantified as described above. The ATPase activity (percent ADP generated in 60 min) was normalized to the amount of ^{35}S-labeled T present in each sample assayed.

To separate monomeric and oligomeric forms of T (9), ^{35}S-labeled extracts (400 μl per gradient) were loaded onto 5 to 20% sucrose gradients containing hypotonic buffer with a 0.2-ml cushion of 70% sucrose. Centrifugation was for 3 h at 58,000 rpm at 2°C with a Beckman SW60Ti rotor. Molecular weight (in thousands [K]) markers included ovalbumin (45K or 3.5S), serum albumin (67K or 4.3S), phosphorylase *b* (dimer = 190K or 7S), catalase (tetramer = 240K or 10S), and 18S and 28S RNA sedimented in parallel. Fractions (5 drops) were collected from the bottom and precipitated with PAb 419 and analyzed by SDS gel electrophoresis. Labeled T bands were quantified as above.

Peptide mapping and phosphoamino acid analysis. ^{32}P-labeled T from 1 × 10^7 to 2 × 10^7 SV-10T or mutant 5080-10T cells was extracted from dried SDS-polyacrylamide gels,

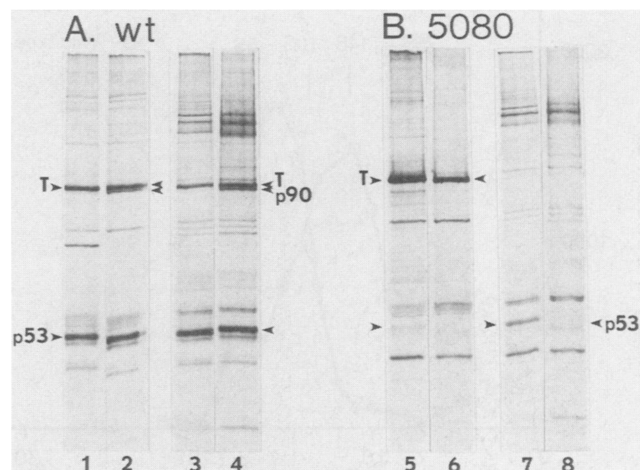

FIG. 2. SDS gel analysis of mutant 5080 and wild-type (wt) T proteins labeled under pulse-chase conditions. SV-10T (A) and 5080-10T (B) cells were labeled with [^{35}S]methionine for 1 h (lanes 1, 3, 5, and 7), followed by a 4-h chase with cold methionine (lanes 2, 4, 6, and 8). Cell extracts were immunoprecipitated with PAb 419 (lanes 1, 2, 5, and 6) and with PAb 122 (lanes 3, 4, 7, and 8) and analyzed on SDS gels as described in Materials and Methods. All lanes shown were autoradiographed for 48 h. All lanes are from the same experiment. The relative positions of labeled T, p90, and p53 bands are shown with arrowheads.

oxidized, and digested with trypsin as previously described (1). Peptides were separated in two dimensions (13) on 100-μm-thick cellulose thin-layer plates by electrophoresis (pH 1.9; 1.5 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 dimension (16). Phosphoamino acid analyses were done by partially hydrolyzing peptides in 5.7 M HCl at 110°C for 1 h and separating the phosphoamino acids on 100-μm-thick cellulose thin-layer 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 dimension (6). Autoradiography was performed by using presensitized Kodak X-Omat AR film.

RESULTS

SDS gel analysis of pulse-chase-labeled 5080 and wild-type T. To measure the relative rate of T synthesis and turnover in SV-10T and mutant 5080-10T cell lines, pulse-chase analysis using [^{35}S]methionine-labeled cells was performed as described in Materials and Methods. Labeled extracts were immunoprecipitated with anti-T (PAb 419) and analyzed by SDS gel electrophoresis and autoradiography. The results for wild-type T and mutant 5080 T are shown in Fig. 2A and B, respectively. By using equivalent amounts of labeled cells, there was about 10-fold more 5080 T than wild-type T after 1 h (Fig. 2, lanes 1 and 5). However, after pulse-chase labeling analysis, 5080 T appeared to be degraded much faster than wild-type T (lanes 2 and 6). For 16-h-labeled T, there was about 0.5- to 0.8-fold as much 5080 T as normal T (results not shown). Thus, there were not appreciable differences in the total amounts of T in these two cell lines. This was confirmed by comparison of the relative amounts of mutant and normal T bands stained with Coomassie blue after SDS gel analysis. On the basis of several experiments, we estimated the approximate half-life for 5080 T to be about 3 h compared with greater than 20 h for wild-type T.

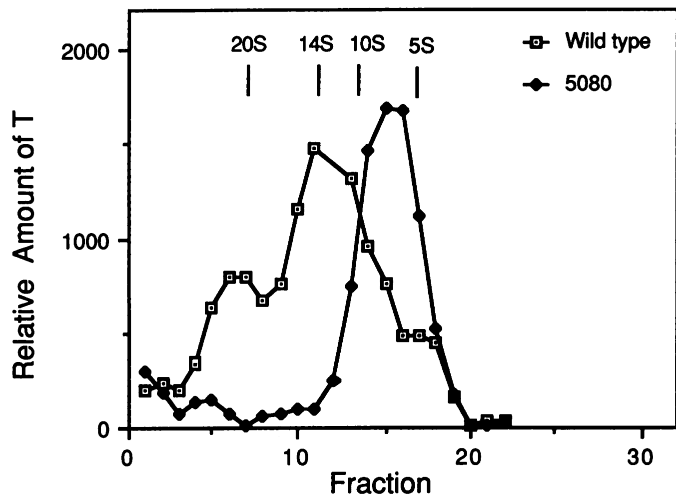

FIG. 3. Gradient fractionation of oligomeric forms of T from mutant 5080-10T (5080) and SV-10T (wild-type) cells. Extracts were sedimented on sucrose gradients as described in Materials and Methods. The relative amounts of ^{35}S-labeled T in each gradient fraction were measured and compared for the two cell lines.

We next compared the relative amounts of mutant and normal T proteins complexed with p53 in the two different cell lines. Figure 2 also shows labeled cell extracts immunoprecipitated with anti-p53 (PAb 122) in parallel with anti-T. For SV-10T cells (Fig. 2A), about 60% of T was bound to p53 after 1 h (lanes 1 and 3), 98% was bound after pulse-chase analysis (lanes 2 and 4), and 90% was bound after 16 h of continuous labeling (results not shown). This is consistent with association of p53 with mature SV40 T molecules (3, 23). The level of p53 in lane 1 is almost equivalent to that in lane 3, consistent with the association of greater than 90% of p53 with T within 30 min after synthesis, as shown for other SV40-transformed cell lines (3, 18). In general, the amount of labeled p53 was about the same as the amount of labeled 94K T band after long labeling times. In contrast, for 5080-10T cells (Fig. 2B), there was essentially no detectable ^{35}S-labeled p53 complexed with 5080 T regardless of labeling time (lanes 5 and 6). This is consistent with previous results for this mutant (15a). The amount of p53 labeled for 1 h and reactive with PAb 122 in 5080-10T cells was about 5 to 10% (lane 7) compared with that of p53 from SV-10T cells (lane 3) where complex formation with SV40 T leads to stabilization of p53 (7). There was almost no detectable p53 after pulse-chase labeling of 5080-10T cells (lane 8). Control untransformed C3H10T1/2 cells reacted with PAb 122 (results not shown), had a similar pattern of labeled bands as that of labeled 5080-10T cells (lanes 7 and 8), and contained about 2 to 5% of the amount of labeled p53 compared with that of SV-10T cell extracts.

With increasing labeling time, an ^{35}S-labeled 90K band (p90) appeared in all immune-complexed T fractions from SV-10T (Fig. 2A), using either anti-T (lane 2) or anti-p53 (lane 4). This 90K band was observed only after pulse-chase labeling (lane 4) or long continuous labeling and not after short 30-min pulse times (lanes 1 and 3). After 12 h, there was about twofold more 94K T than p90 in immune-complexed wild-type cell extracts. In contrast, no detectable p90 or p53 was precipitated from 5080-10T cell extracts with anti-T (lane 6). The same result was observed for another anti-T, PAb 109. When short or long labeled 5080-10T extracts were reacted with anti-p53 (lane 8), a trace amount of a 90K band appeared to be present in the immune complexes (lanes 7 and 8) that was not present in samples precipitated with a nonspecific NS1 antibody (results not shown). However, it is possible that the faint 90K band in lanes 7 and 8 is not the same as the p90 band in lanes 2 and 4 and may be a different specific protein bound to p53 or a nonspecific "sticky" protein. There was little (if any) detectable 90K protein immunoprecipitable from short or long labeled parental untransformed C3H10T1/2 cells (results not shown).

ATPase and oligomerization analysis. The ATPase activities of immune-complexed mutant 5080 and normal T fractions were analyzed as described in Materials and Methods. The rate of hydrolysis of [α-^{32}P]ATP to [α-^{32}P]ADP per microgram of T per min was calculated (data not shown). Immune-complexed wild-type T from SV-10T cells hydrolyzed ATP at a similar rate as previously reported for SV40 T from lytically infected monkey cells (22). However, 5080 T had essentially no detectable ATPase activity, as its relative specific activity was 0.06 compared with 1.0 for normal T.

The distribution of monomeric and oligomeric species of mutant and normal T was measured after gradient fractionation. Cells were labeled with ^{35}S for 16 h, and hypotonic extracts were centrifuged on 5 to 20% sucrose gradients. The distribution of labeled monomeric and oligomeric T forms was determined as described in Materials and Methods. The results are shown in Fig. 3. Extracts from SV-10T cells consisted predominantly of 14S (tetrameric) and 20S forms of T as previously predicted (9). In contrast, extracts from 5080-10T cells contained only 5 to 10S species of T, with no

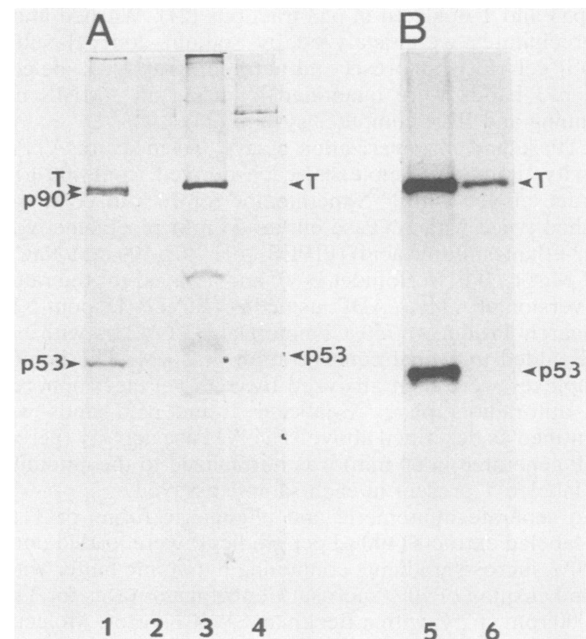

FIG. 4. Phosphorylation analysis of mutant 5080 and normal T complexes. SV-10T and 5080-10T cells were labeled for 4 h in vivo with [^{35}S]methionine (A) and $^{32}P_i$ (B), immunoprecipitated, and analyzed on SDS gels. (A) SV-10T (lanes 1 and 2) and 5080-10T (lanes 3 and 4) extracts were sequentially immunoprecipitated with anti-T PAb 109 (lanes 1 and 3), followed by anti-p53 PAb 122 (lanes 2 and 4). (B) SV-10T (lane 5) and 5080-10T (lane 6) cell extracts were reacted with anti-T only. Exposures for ^{35}S- and ^{32}P-labeled gels were for 3 h at room temperature and −70°C, respectively, without an intensifying screen.

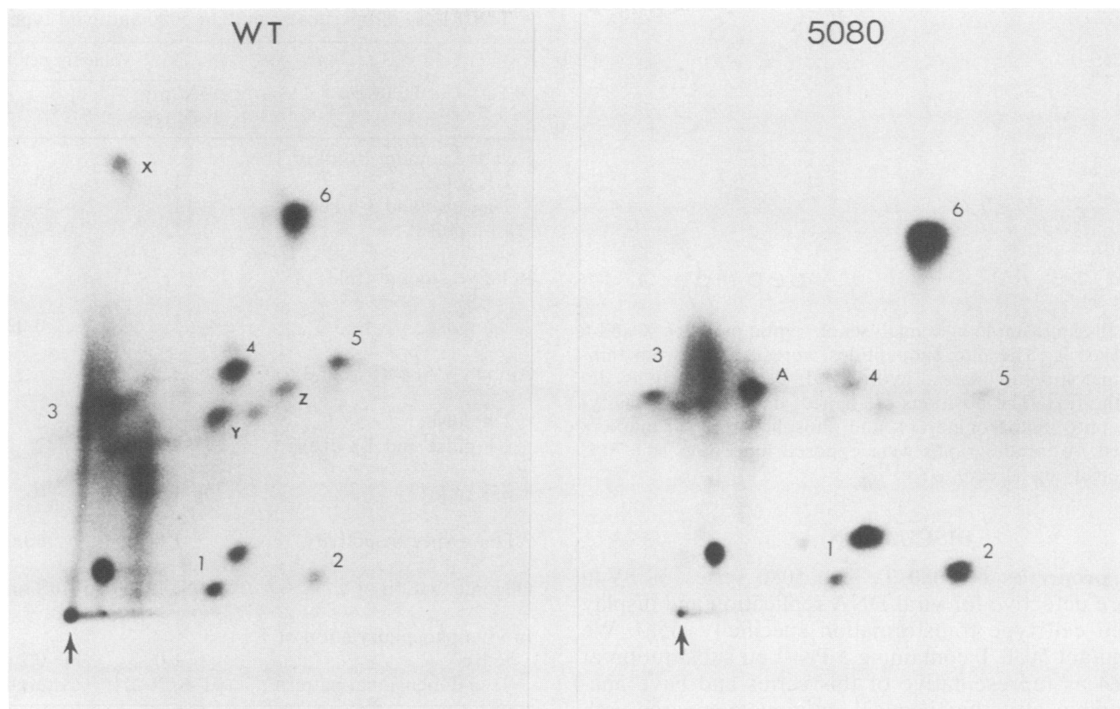

FIG. 5. Two-dimensional phosphopeptide analysis of mutant 5080 and wild-type (WT) T. Tryptic digests of ^{32}P-labeled 90K to 94K T bands were analyzed by electrophoresis at pH 1.9 (horizontal direction) and chromatography (vertical direction) as described in Materials and Methods. The origin is indicated with a vertical arrow. Equal counts (1,500 Cerenkov cpm) were loaded on each plate. Autoradiographs shown were exposed for 2 days at −70°C with an intensifying screen.

detectable 14S or 20S oligomeric forms present. We conclude that 5080 T is defective in its ability to form oligomers greater than a monomer or dimer.

In vivo phosphorylation sites. We have observed a relationship between bound p53 and altered phosphorylation of both T and p53 in complex from lytically infected CV1 cells (L. C. Tack and J. H. Wright, unpublished data). Thus, we wanted to analyze the specific in vivo phosphorylation of wild-type and mutant T expressed in SV-10T and 5080-10T cells, respectively. Cells were labeled for 4 h in vivo with [^{35}S]methionine and ^{32}P as described in Materials and Methods. Equal volumes of labeled cell extracts were then immunoprecipitated and analyzed on SDS gels. Figure 4A shows the results for ^{35}S-labeled SV-10T (lanes 1 and 2) and 5080-10T (lanes 3 and 4) cell extracts sequentially immunoprecipitated with the anti-T PAb 109 (lanes 1 and 3), followed by the p53-specific PAb 122 (lanes 2 and 4). As in Fig. 2, there was no detectable ^{35}S-labeled p53 associated with 5080 T (Fig. 4, lane 3); however, very small amounts of free p53 were present in the 5080 anti-T supernatant and reactive with PAb 122 (lane 4). Figure 4B shows the results for equal volumes of ^{32}P-labeled SV-10T (lane 5) and 5080-10T (lane 6) cell extracts immunoprecipitated with anti-T. Again, there was no ^{32}P-labeled p53 associated with SV40 T in the 5080 sample (lane 6). The relative specific activity (ratio of ^{32}P/^{35}S) for 5080 T was significantly less (0.1 to 0.4) than for normal T (1.0). Under conditions in which the ^{32}P-labeled 94K T and p90 bands were better resolved in one-dimensional SDS gels, p90 appeared to be phosphorylated in the T+p53 complex from SV-10T cells (results not shown).

To compare the phosphorylation sites of wild-type and mutant T, ^{32}P-labeled proteins were extracted from SDS gels, oxidized, and digested with trypsin and equivalent amounts (measured by equal counts per minute) of radiolabeled phosphopeptides were separated in two dimensions by electrophoresis and chromatography. Tryptic digestion of normal T from SV-10T cells generated the phosphopeptide pattern shown in Fig. 5. The known peptides are numbered according to Scheidtmann et al. (16). Peptides 1, 2, and 4 represent overlapping peptides, all of which are phosphorylated at Ser-639. Peptide 3 is an amino-terminal peptide containing phosphoserine and phosphothreonine. This peptide contains a different number of basic residues at its C terminus, thus generating a smear in the positive direction. Peptide 5 is a carboxy-terminal peptide that can be phosphorylated on at least two serines. Peptide 6 is phosphorylated at Thr-701. Three additional peptides, X, Y, and Z, were present and have not previously been described.

Tryptic digestion of ^{32}P-labeled mutant T from 5080-10T cells also generated peptides 1 through 6. A mix confirmed this (data not shown). However, peptides 3 and 5 were less phosphorylated for 5080 T compared with normal T. The sum of peptides 1, 2, and 4 (the partially digested peptides contained the same Ser-639 site) was similar for the two forms of T. Phosphopeptides X, Y, and Z were absent from digests of the mutant protein. A new peptide, A, not present in the wild-type digest, consistently appeared in the 5080 T digest. Peptide A is not well resolved from peptide 3 in the long exposure shown in Fig. 5; however, shorter exposures clearly show it to be a distinct 5080-specific peptide.

The labeled peptides were extracted and analyzed for their phosphoamino acid content. Peptides 1, 2, 4, and 5 (Fig. 6) contained phosphoserine, peptide 6 contained phosphothreonine, and peptide 3 contained both phosphoserine and phosphothreonine. These results are in agreement with those of Scheidtmann et al. (16). Peptide X contained phosphothreonine (Fig. 6), and peptides Y, Z, and A contained phosphoserine (data not shown).

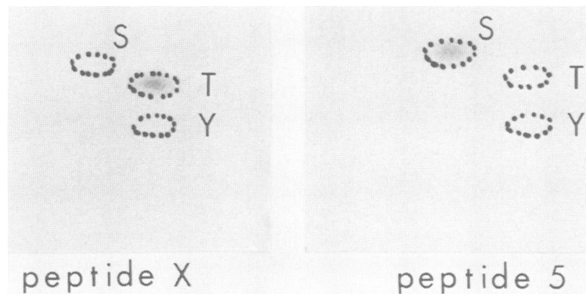

FIG. 6. Phosphoamino acid analysis of tryptic peptides X and 5 from wild-type T. The phosphopeptides were isolated from thin-layer chromatographic plates, hydrolyzed, and analyzed as described in the text. The positions of stained, unlabeled phosphotyrosine (Y), phosphothreonine (T), and phosphoserine (S) markers are indicated. Autoradiographs were exposed for 5 days at −70°C with an intensifying screen.

DISCUSSION

Altered properties of 5080 T. The 5080 series of SV40 mutants are defective for viral DNA replication and display a restricted cell type transformation specificity (15a). We chose a mutant 5080 T containing a Pro-Leu substitution at residue 584 as representative of this series and have analyzed a number of its biochemical activities compared with those of the wild-type protein. Two different clonal variants of 5080-10T cell lines were examined with essentially identical results.

A summary of our data is shown in Table 1. Mutant 5080-10T cells expressed an altered T that was synthesized at a significantly higher (10-fold) rate but with lower stability than normal T expressed in SV-10T cell lines. Wild-type T from SV-10T cell extracts was complexed with the p53 protein, while 5080 T was not associated with p53. The levels of p53 were much lower in the 5080-10T cell line, consistent with previous observations that SV40 T stabilizes p53 and p53 stabilization is not required to transform C3H10T1/2 cells (7, 15a, 20) 5080 T lacked detectable ATPase activity. Sucrose gradient fractionation of labeled 5080 T indicated it was unable to oligomerize beyond the 5 to 10S form to 14S or greater; normal T sedimented as predominantly 14S and 20S oligomers. The predominant 7S species of 5080 T is thus equivalent to a monomer or dimer species of T. In addition, the in vivo phosphorylation level of 5080 T was significantly decreased compared with that of wild-type T. Phosphopeptide analysis of 5080 T indicated an altered pattern; the N-terminal peptide 3 containing serines 106, 111, 112, and 123 and threonine 124 as well as the C-terminal peptide 5 (with previously identified phosphoserines 676 to 679) were less phosphorylated than the corresponding peptides from wild-type T. Phosphopeptides X, Y, and Z were present in wild-type T but lacking in 5080 T. The source of peptides X, Y, and Z is not yet clear.

A 90K protein that we have named p90 coprecipitated with both anti-T and anti-p53 monoclonal antibodies and was present in T complexes from SV-10T but not 5080-10T or control C3H10T1/2 cells regardless of labeling time. p90 appeared to be associated with normal T+p53 complexes after long labeling times. The p90 protein may be an altered form of T or a cellular protein bound to T+p53 complexes. p90 may be a heat shock protein such as hsp90. p53 is known to bind to the heat shock protein hsp70 in an ATP-dependent manner (5). One or more of phosphopeptides X, Y, and Z may be contributed by p90.

TABLE 1. Properties of mutant 5080 and wild-type SV40 T's

T property	Value for cell line		
	SV-10T (wild type)	5080-10T	Control 10T
Relative amount (fold) of T[a]			
1-h pulse	1	10	0
1-h pulse and 3-h chase	1	2–5	0
16-h pulse	1	0.5–0.8	0
Relative amount (fold) of p53[a]			
1-h pulse	1	0.02	0.01
Amount (%) of T bound to p53[a]			
1-h pulse	61	<2	0
1-h pulse and 4-h chase	98	<2	0
T/p53 ratio[a]	1.0	>30	ND
ATPase specific activity	1.0	0.06	0
Oligomerization of T	20S, 14S	5–10S only	ND
In vivo phosphorylation of T			
Pulse[b]	1.0	0.2	0
Two-dimensional pattern	p1–6, X–Z	Altered	ND

[a] Based on [^{35}S]methionine-labeled 94K T and p53 bands.
[b] Specific activity measured as ^{32}P/^{35}S ratio.

Interrelationships among the biochemical properties of wild-type and 5080 T. What is the relationship between these various changes in the mutant T, i.e., what is the time-dependent sequence of events and is it possible to evaluate their order of importance? For example, the higher rate of T synthesis in 5080-10T cells compared with that in SV-10T cells suggests altered or even defective binding to the SV40 *ori* region. Although the DNA-binding domain in T is not thought to be conformationally sensitive, 5080 T may be unable to bind to SV40 *ori* sequences and autoregulate early RNA synthesis. Even if 5080 T were competent for *ori* binding, T lacking bound p53 and T complexed with p53 from SV40-infected CV1 cells have been shown to bind at different positions within the SV40 *ori* region (21). Since most T from SV-10T cells is complexed with mouse p53 while 5080 T lacks bound p53 (Fig. 2; 15a), their respective binding to *ori* is expected to be altered. Thus, the amounts of early SV40 RNA synthesized may be quite different in the two cell lines. Other possibilities include altered RNA stability or translational efficiency. Regardless, enhanced 5080 T synthesis appears to compensate for its greater instability, as the resulting steady-state level of 5080 T is similar to the level of wild-type T in SV-10T cells.

p53 binding, oligomerization, and increased phosphorylation are all associated with later events leading to the normal maturation of newly synthesized T. In fact, one or more of these age-related changes may be responsible for the high level of stability observed for the normal T protein. T oligomerizes (with a half-time of about 1 h in SV40-infected cells) into a predominantly tetrameric form after it migrates into the nucleus (9, 17). The association of p53 with SV40 T occurs in the nucleus within 5 to 30 min after synthesis (3, 18). Therefore, a half-life of 3 h for 5080 T appears sufficient for both oligomerization and p53 binding to occur before degradation, suggesting that instability of 5080 T cannot be responsible for defects in oligomerization and p53 binding

but may affect the phosphorylation pattern. The temporal sequence for oligomerization, p53 binding, and phosphorylation is not clear. All are affected in the mutant 5080 T, but determination of the degree of interrelatedness of these three maturation-related events requires further analysis.

It should be noted that the phosphorylation pattern of wild-type T (>90%) complexed with mouse p53 from transformed C3H10T1/2 cells is identical to the small fraction of lytic T complexed with monkey p53; additionally, the phosphorylation pattern for 5080 T (defective for mouse p53 and p90 binding) is essentially identical to that of lytic T depleted in bound monkey p53 and p90 by sequential immunoprecipitation (J. H. Wright, C. Frankel, and L. C. Tack, submitted for publication). Thus, the altered phosphorylation pattern for T from SV-10T cells compared with that from 5080-10T cells appears to be due to p53 complex formation and not species-specific or transformation-dependent phosphorylation events.

We have previously observed a relationship between ATPase activity and p53 binding for complexes of T from lytically infected monkey cells (21, 22). However, neither defects in p53 binding, phosphorylation, oligomerization, nor stability appear to directly account for the loss of ATPase activity of mutant 5080 T based on the following observations: (i) newly synthesized T is depleted in p53 and is a highly ATPase-active form of T (22, 23); (ii) phosphorylation appears to have little effect on the ATPase activity of purified SV40 T expressed in cell line 293 (14); and (iii) monomeric T is a better ATPase than oligomeric T (2). It is most likely that loss of ATPase activity is a consequence of gross structural changes that occur in the 5080 T molecule at early times after synthesis. Phosphorylation, p53 binding, and perhaps oligomerization events, while not required for ATP binding and hydrolysis, may instead be important factors in regulating the activity of this domain in SV40 T.

Summary. There are a number of biochemical changes that distinguish the mutant 5080 T protein from its wild-type T counterpart: lack of p53 binding, decreased stability, altered phosphorylation, lack of oligomerization, and loss of ATPase activity. That so many properties are affected by a single substitution in T emphasizes the importance of the small hydrophobic domain in modulating higher-order structure in SV40 T.

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