JOURNAL OF VIROLOGY, Apr. 1991, p. 2066–2072
0022-538X/91/042066-07$02.00/0

Vol. 65, No. 4

Stable T-p53 Complexes Are Not Required for Replication of Simian Virus 40 in Culture or for Enhanced Phosphorylation of T Antigen and p53

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Received 30 August 1990/Accepted 18 December 1990

We generated a number of simian virus 40 (SV40) mutants with single amino acid substitutions in T antigen between residues 388 and 411. All but one mutant (398LV) replicated like wild-type SV40 and gave rise to normal-size plaques. Three different mutations at residue 402 (Asp to Glu, Asn, or His) totally prevented the formation of stable complexes with the cellular protein p53 in monkey cells but had no effect on virus replication. Only one other mutation in this region, involving residue 401 (Met to Thr), slightly inhibited the formation of T-monkey p53 complexes. The three mutant T antigens with substitutions at residue 402 also formed no stable complexes with human p53 but generated low levels of complexes with mouse p53. These results indicate that residue 402 is critical for binding to monkey and human p53 proteins and is important for binding to mouse p53. We suggest that it is one of several points of contact. In cells infected with any one of the three residue 402 mutant viruses, T antigen and p53 became increasingly phosphorylated, as they were in cells infected with wild-type virus. Our data therefore show that stable T-p53 complexes are not required for replication of SV40 in culture or for enhanced phosphorylation of either protein.

The large tumor (T) antigen of simian virus 40 (SV40) is a multifunctional phosphoprotein that is required for productive viral infection (48, 53) and for cell transformation (5, 16, 23, 31, 49). In infected cells, T antigen initiates the replication of virus DNA (10, 27). At least four biochemical activities of T antigen are required for SV40 DNA synthesis: origin-specific DNA binding (36, 38, 51), DNA melting (3), ATPase (13, 52), and DNA helicase (44).

The cellular phosphoprotein p53 is found in a complex with T antigen in both SV40-infected and SV40-transformed cells (14, 24). At the present time, it is not known whether this association is necessary for viral replication or for transformation. There are indications that p53 is involved in lytic infection, because mouse p53 can inhibit the binding of T antigen to DNA polymerase α (12) and interfere with T-antigen-directed SV40 DNA synthesis in vitro (4, 55). It has been suggested that monkey p53 modulates a number of biochemical activities of T antigen in vitro (47).

Several investigators have generated mutants of T antigen which fail to bind to p53 (43, 46, 50). For the most part, these mutant proteins contain deletions or nonconservative substitutions which adversely affect various biochemical activities of T antigen and are consequently lethal to the virus. It has therefore been difficult to reach conclusions about the requirements for p53 in productive infection or its possible role.

During the course of infection in monkey cells, T antigen and p53 become increasingly phosphorylated (15, 34, 37, 39, 45). Both proteins are substrates of cdc2 kinase in vitro (2, 26), and p53 is loosely associated with cdc2 kinase in vivo (28), suggesting that both proteins are substrates for cdc2 kinase in vivo as well. While phosphorylation positively and negatively regulates T antigen's activities (26, 29, 32, 37, 39, 54), no function has yet been attributed to p53 phosphorylation. It is likely to be of importance to the virus since T antigen induces or activates a protein kinase to phosphorylate p53 in SV40-infected and SV40-transformed cells (34). This observation raises the question of whether T antigen needs to physically associate with p53 to induce such a kinase.

In order to investigate the role of T-p53 complexes in productive infection, we generated a number of single-point mutations between residues 388 and 411 of T antigen and tested the effects of each mutation on virus replication and binding to p53. The p53-binding region on T antigen has been previously localized to residues 272 to 517 (35), although a smaller region may be involved. Residues 388 to 411 were chosen partially because this region lies within the epitopes recognized by all monoclonal antibodies that inhibit the association of T antigen with p53 (18). Furthermore, this region maps between the zinc finger (22) and ATPase (7) regions and contains no known activity necessary for virus replication. We found that one site (Asp-402) is important for the stable binding to monkey, human, and mouse p53. Furthermore, stable T-p53 complexes are not required for virus replication in culture or for the enhanced phosphorylation of T antigen and p53 during productive infection.

MATERIALS AND METHODS

Plasmid and mutagenesis protocol. pBS-SV40 consists of a Bluescript SK(+) vector (Stratagene) and the entire SV40 genome ligated at their *Bam*HI sites (22). Mutations were generated in pBS-SV40 by annealing oligonucleotides with one mismatch to uridine-containing single-stranded DNA templates as previously described (40). The oligonucleotide was extended with T4 DNA polymerase, and the resulting double-stranded DNA was sealed with T4 DNA ligase. The DNA was used to transform *Escherichia coli* BMH 71-18 (International Biotechnologies, Inc.). Single-stranded DNA was prepared and sequenced by the dideoxy procedure (33) using appropriate primers.

Viral replication assays. pBS-SV40 bearing a mutation in

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the T-antigen gene was cleaved with *Bam*HI to release the mutant genomic DNA. The DNA was ligated at a low DNA concentration to favor self-ligation of the SV40 genome. Circular DNA was transfected into monkey BSC-1 cells by the DEAE-dextran procedure (25). Plaques were visualized by staining with neutral red (48) and counted 10 to 14 days posttransfection. Plates which did not have any plaques were incubated for a minimum of 4 weeks to make sure that small plaques did not appear.

Cells and transfections. Mouse BALB/c 3T3 cells (1) and human skin fibroblasts were obtained from the American Type Culture Collection. These two cell lines were maintained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum. BSC-1 cells are an established line of African green monkey kidney cells (Flow Laboratories, Inc.) and were maintained in Dulbecco's modified Eagle's medium containing 7% calf serum. Subconfluent BSC-1 cells in 35-mm-diameter dishes were transfected by the DEAE-dextran procedure (25) as previously described (40).

Viruses and infections. Viable mutant virus was obtained by lifting individual plaques. Virus was propagated in BSC-1 cells to obtain working stocks. Virus stocks were titered and used to infect BSC-1 cells at 10 PFU per cell and human or mouse cells at 100 PFU per cell.

Immunoprecipitation and gel electrophoresis. Cells infected with wild-type or mutant virus were labeled with L-[^{35}S]methionine (200 μCi/ml) at 41 h postinfection (p.i.) for 4 h. For the phosphorylation experiments, infected cells were labeled with $^{32}P_i$ (250 μCi/ml) at 16 or 41 h p.i. for 4 h. Cells were lysed with Nonidet P-40 lysis buffer (20), and labeled T antigen or T-p53 complexes were immunoprecipitated with anti-T monoclonal antibody pAb416 (14). p53 and bound T antigen were immunoprecipitated with p53-specific antibody pAb421 (14), pAb246 (56), or pAb248 (56). Labeled immunoprecipitated proteins were analyzed by sodium dodecyl sulfate (SDS) gel electrophoresis and autoradiography. X-ray films were scanned with an LKB densitometer to quantitate the intensity of T-antigen and p53 bands.

TABLE 1. Virus replication and T-monkey p53 complexing assays of mutants with single amino acid substitutions in T antigen

Amino acid position	Amino acid change		Mutant name	Plaque formation[a]	Relative % of p53 bound to T antigen[b]	Relative % of T antigen bound to p53[c]
	From	To				
None[d]				+	100	100
388	Met	Leu	388ML	+	180	80
390	Gly	Ala	390GA	+	260	145
392	Ala	Gly	392AG	+	195	91
394	Leu	Val	394LV	+	149	79
395	His	Asn	395HN	+	112	91
396	Cys	Ser	396CS	+	270	158
397	Leu	Val	397LV	+	95	90
398	Leu	Val	398LV	−	81[e]	98[e]
399	Pro	Leu	399PL	+	159	110
400	Lys	Arg	400KR	+	91	101
400	Lys	Glu	400KE	+	220	98
401	Met	Thr	401MT	+	39	43
402	Asp	Glu	402DE	+	0	0
402	Asp	Asn	402DN	+	0	0
402	Asp	His	402DH	+	0	0
403	Ser	Ala	403SA	+	79	107
404	Val	Leu	404VL	+	78	101
407	Asp	Glu	407DE	+	110	104
408	Phe	Tyr	408FY	+	100	93
410	Lys	Arg	410KR	+	110	59
411	Cys	Arg	411CR	+	91	110

[a] +, Mutant DNA gave rise to about the same number of plaques (within a factor of 2) as wild-type SV40; −, mutant did not give rise to viral plaques and value represents a difference of a factor of at least 2×10^3 in titer.

[b] Percentage of p53 coimmunoprecipitated with mutant T antigen (pAb416 reaction) relative to that precipitated with wild-type T antigen.

[c] Percentage of mutant T antigen that was coimmunoprecipitated with p53 (pAb421 reaction) relative to wild-type T antigen.

[d] Wild-type SV40 used.

[e] The T-p53 complexing assay of this nonviable mutant was performed by transfection of BSC-1 cells with mutant SV40 DNA by using a DEAE-dextran procedure. For purposes of calculating percentages, cells were also transfected with wild-type DNA.

RESULTS

Generation of mutations in a potential p53-binding region of T antigen. We generated a series of mutant T antigens with conserved, single amino acid substitutions between residues 388 and 411 to identify possible p53 contact sites (Table 1). Conservative substitutions were chosen for most residues in order to keep structural alterations to a minimum. A similar strategy has been used by us to study the organization of the DNA-binding domain of T antigen (38, 40, 41). Mutations were generated in a plasmid containing the entire SV40 genome (pBS-SV40) (22). The mutagenesis protocol was similar to the one devised by Kunkel (17) and has been previously described (22).

Assays for virus replication in permissive monkey cells. Mutant SV40 DNAs were excised from bacterial plasmids (pBS-SV40) and religated at low DNA concentrations to favor self-ligation of the SV40 genome. The viral DNA was transfected into permissive monkey BSC-1 cells to test the effect of each mutation on the formation of plaques. Table 1 shows a summary of the results of these plaque assays for wild-type and mutant DNAs. Nearly all mutants replicated like wild-type SV40 and gave rise to normal-sized plaques (Table 1). This indicates that most of this region is not critical for virus replication functions. One mutant (398LV) did not replicate under the conditions tested. Mutant 399PL is temperature-sensitive in that it replicated at 37 and 33°C but not at 40°C (data not shown). The significance of these two adjacent sites (residues 398 and 399) in viral replication is unknown. Mutations in this small region are likely to affect the oligomerization (30), DNA polymerase α binding (42), or ATPase function (7) of T antigen. The cysteine residues at 396 and 411 may participate in disulfide bonds. However, mutations at these sites did not have an effect on viral replication.

T-p53-complexing assays in monkey, human, and mouse cells. We determined whether mutant T antigens formed complexes with p53 in monkey BSC-1 cells. The p53 in this cell line is wild type because it is identical in sequence to the p53 of primary monkey cells (32a). Replication-competent mutants were tested by infecting cells with virus recovered from individual plaques. The single replication-negative mutant was tested by transfection with viral DNA. Cells were labeled with L-[^{35}S]methionine, and T-p53 complexes were identified by immunoprecipitation reactions with anti-T and anti-p53 monoclonal antibodies. Examples of results of typical complexing assays are shown in Fig. 1. To quantitate our results, X-ray films were traced by densitometry, and we calculated the percentage of labeled mutant T antigen bound to p53 relative to that of wild-type T antigen and, conversely, the percentage of labeled p53 bound to mutant T antigen relative to that bound to wild-type T antigen (Table 1). In the

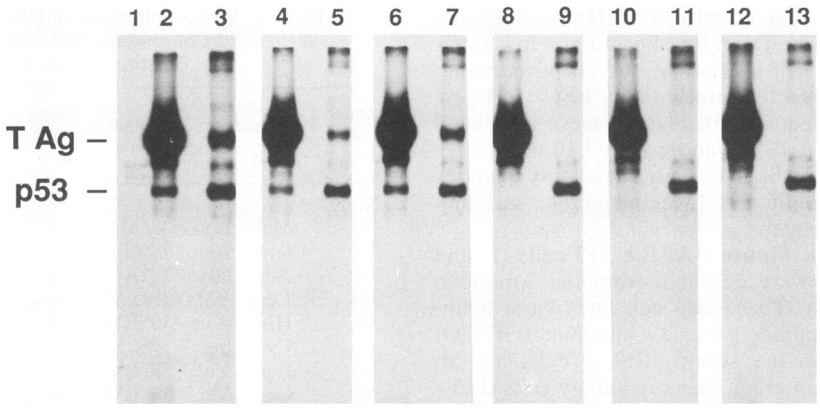

FIG. 1. Association of wild-type or mutant T antigen with p53 in infected monkey BSC-1 cells. Cells were infected with wild-type or mutant SV40 and labeled with L-[^{35}S]methionine for 4 h. Cell lysates were incubated with either pAb416 (14), a monoclonal antibody to T antigen, or pAb421 (14), a monoclonal antibody to p53. Labeled immunoprecipitated proteins were detected by autoradiography after electrophoresis on SDS–13% polyacrylamide gels. Lanes 1 through 3, Wild-type SV40; lanes 4 and 5, 401MT; lanes 6 and 7, 403SA; lanes 8 and 9, 402DE; lanes 10 and 11, 402DN; lanes 12 and 13, 402DH. The following antibodies were used: normal hamster serum (lane 1), pAb416 (lanes 2, 4, 6, 8, 10, and 12), and pAb421 (lanes 3, 5, 7, 9, 11, and 13).

first round of mutagenesis, only one mutant T antigen with a substitution at residue 402 (Asp to Glu) formed no detectable stable complexes with monkey p53 (Fig. 1, lanes 8 and 9, and Table 1). Reactions with anti-T monoclonal antibody pAb416 precipitated both T antigen and its bound p53 from cells infected with wild-type SV40 and mutants 401MT and 403SA (Fig. 1, lanes 2, 4, and 6, respectively) but precipitated only T antigen from cells infected with mutant 402DE (Fig. 1, lane 8). This result was confirmed by immunoprecipitation reactions with anti-p53 monoclonal antibody pAb421. In this case, p53 and its bound T antigen were precipitated from cells infected with wild-type SV40 and mutants 401MT and 403SA (Fig. 1, lanes 3, 5, and 7, respectively), but only p53 was precipitated from cells infected with 402DE (Fig. 1, lane 9). All other mutations had little or no adverse effect on the T-p53 binding reaction, with the exception of the substitution at residue 401 (Met to Thr), which reduced binding to a small degree (Table 1 and Fig. 1, lanes 4 and 5). Interestingly, although the Leu-to-Val substitution at residue 398 abolished the ability of the virus to replicate, it did not affect the ability of T antigen to bind to p53.

To test the importance of residue 402 in the association of T antigen with p53, we made two additional, less conservative mutations at this site (Asp to Asn and to His). The two new virus mutants also replicated as well as wild-type SV40 (Table 1). However, their T antigens did not form detectable complexes with p53 in BSC-1 cells (Fig. 1, lanes 10 through 13, and Table 1). This demonstrates that Asp-402 is an important residue for binding to monkey p53. Although complexes were not detected with any of the residue 402 mutant T antigens, we cannot exclude the possibility that there may have been unstable or short-lived complexes in vivo. Nevertheless, these results indicate that the formation of stable T-p53 complexes is not required for the replication of SV40 in cultured monkey cells.

We next investigated whether the mutants with substitutions at residue 402 could bind to p53 in human fibroblasts. No stable T-human p53 complexes were detected by immunoprecipitation reactions (Table 2). Therefore, we conclude that this residue is also essential for binding to human p53.

Complexes between T antigen and rodent p53 are more stable than those involving primate p53 (19), possibly indicating a greater number of contact points. Therefore, we examined whether Asp-402 is also important for stable binding to mouse p53. Mouse BALB/c 3T3 cells were abortively infected with wild-type or mutant virus. After infection, these cells transiently express a functional large T antigen (9). The p53 of this cell line is recognized by antibody pAb246, suggesting that it is similar to wild-type p53 in its ability to bind to T antigen (21). Infected cells were labeled (for 3 h) with L-[^{35}S]methionine, and T-p53 complexes were detected by immunoprecipitation reactions. When cells were infected with wild-type virus, most or all of the p53 was coimmunoprecipitated with T antigen (Fig. 2, lanes 2 through 4). However, reduced amounts of p53 were coimmunoprecipitated from cells infected with any of the residue 402 mutant viruses (Fig. 2, lanes 5 through 13). Quantitative results are shown in Table 2. The substitution of Asp with Glu at residue 402 had some effect on the binding of T antigen to mouse p53 (Table 2). More severe effects were noted when Asp-402 was substituted with Asn or His. These results indicate that this residue is important for binding to mouse p53, although it is not as essential as it is for stable binding to monkey or human p53.

Phosphorylation of T antigen and p53 during infection with wild-type or mutant SV40. During infection of permissive monkey cells with wild-type SV40, T antigen and p53

TABLE 2. Binding of mutant T antigens to human and mouse p53[a]

Virus	Human skin fibroblasts		Mouse BALB/c 3T3 cells	
	Relative % of p53 bound to T antigen	Relative % of T antigen bound to p53	Relative % of p53 bound to T antigen	Relative % of T antigen bound to p53
WT SV40[b]	100	100	100	100
402DE	0	0	43	21
402DN	0	0	12	8
402DH	0	0	3	19

[a] Labeled T antigen and p53 were immunoprecipitated from infected cells. Relative percentages were calculated as described in Table 1, footnotes *b* and *c*.

[b] WT, Wild type.

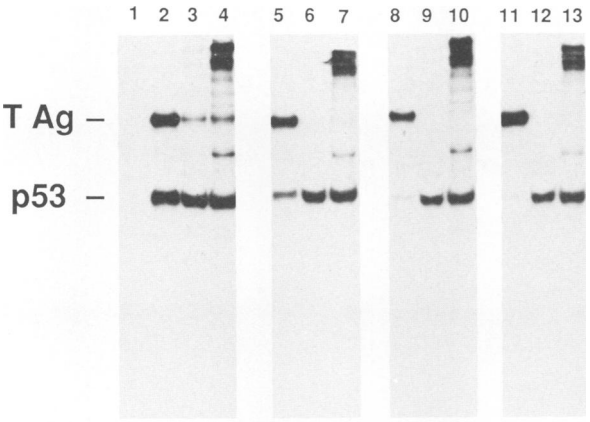

FIG. 2. Immunoprecipitations of T antigen and p53 from mouse BALB/c 3T3 cells abortively infected with wild-type or mutant SV40. At 45 h p.i., cells were labeled with L-[^{35}S]methionine for 3 h. Labeled cell lysates were incubated with anti-T monoclonal antibody pAb416 (lanes 2, 5, 8, and 11) or anti-mouse p53 monoclonal antibody pAb246 (56) (lanes 3, 6, 9, and 12) or pAb248 (56) (lanes 4, 7, 10, and 13). Immunoprecipitates were analyzed by SDS gel electrophoresis. Lanes 1 through 4, Wild-type SV40; lanes 5 through 7, 402DE; lanes 8 through 10, 402DN; lanes 11 through 13, 402DH.

become increasingly phosphorylated (15, 34, 37, 45). There is some evidence that T antigen induces or activates a protein kinase to phosphorylate p53 (34). The presence of stable T-p53 complexes in productive infection raises the question of whether this complex is required for the increased phosphorylation of these proteins. To address this question, we compared the phosphorylation of p53 and T antigen in BSC-1 cells infected with wild-type SV40 with the phosphorylation obtained after infection with the residue 402 mutants. Infected cells were labeled with L-[^{35}S]methionine and, separately, with $^{32}P_i$ at various times after infection. T antigen and p53 were isolated by immunoprecipitation and separated by SDS-polyacrylamide gel electrophoresis (Fig. 3 and 4). The ratios of ^{32}P to ^{35}S in T antigen and p53 were calculated to determine the levels of phosphorylation of these proteins (Table 3). Because the level of phosphorylation of p53 in mock-infected BSC-1 cells remained relatively constant throughout the time course (Fig. 3A, lanes 1 through 3), we were able to calculate the changes induced by infection with wild-type or mutant virus. At 20 h p.i., there was no significant change in the level of phosphorylation of p53 in cells infected with wild-type or mutant SV40 (Fig. 3, lanes 5, 8, and 11, and Table 3). At 45 h p.i., the phosphorylation level of p53 increased significantly in all virus-infected cells (Fig. 3, lanes 6, 9, and 12, and Table 3). These results show that substitutions at residue 402 do not significantly affect the phosphorylation of p53 in infected cells, even though stable T-p53 complexes do not form.

Figure 4 shows the change in phosphorylation of T antigen during infection with wild-type or mutant virus. Quantitation of these results (Table 3) shows that phosphorylation of T antigen increases about twofold during infection with wild-type virus. Similar increases were noticed in cells infected with mutant viruses (Table 3). These results show that the enhanced phosphorylation of T antigen is also not dependent on the formation of stable T-p53 complexes.

DISCUSSION

In this paper, we showed that T antigens with various substitutions at residue 402 were unable to form stable complexes with monkey and human p53. The three mutant viruses all formed approximately the same size and number of plaques as wild-type SV40. These results imply that the stable association of these two proteins is not required for virus replication in culture. However, we cannot exclude the possibility that unstable or short-lived T-p53 complexes form in vivo and are involved in the replication of SV40. It is also possible that stable T-p53 complexes are not required for virus replication in monkey cell cultures but are required for

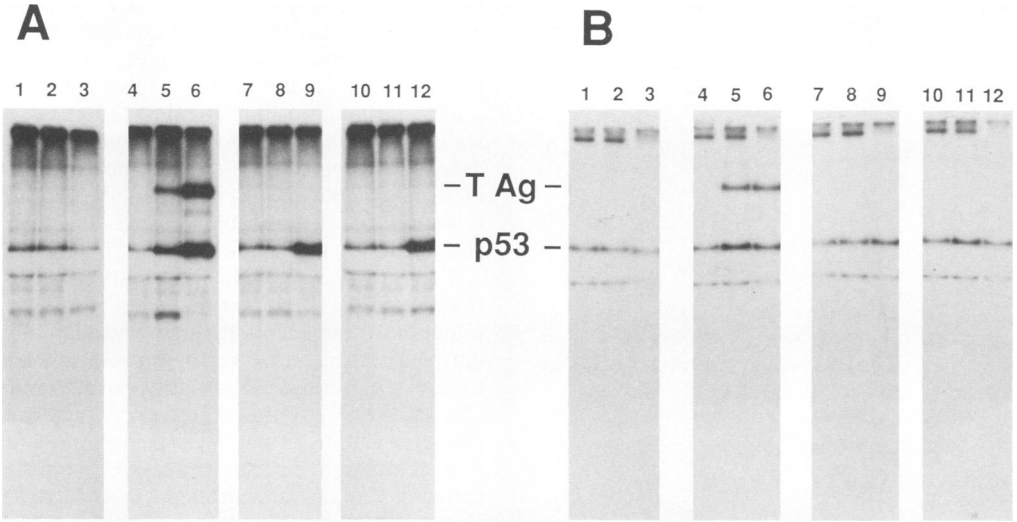

FIG. 3. Phosphorylation of p53 during productive infection of BSC-1 cells. Subconfluent monolayers of BSC-1 cells were infected with wild-type SV40 or with mutant 402DN or 402DH or were mock infected. The cells were labeled with [^{32}P]phosphate (A) or L-[^{35}S]methionine (B) for 3 h. At 0, 20, and 45 h p.i., cells were lysed and the lysates were incubated with anti-p53 monoclonal antibody pAb421. Labeled proteins were analyzed on 13% polyacrylamide gels and detected by autoradiography. Lanes 1 through 3, Mock infection; lanes 4 through 6, wild-type SV40; lanes 7 through 9, 402DN; lanes 10 through 12, 402DH. Cells were harvested at 0 h (lanes 1, 4, 7, and 10), 20 h (lanes 2, 5, 8, and 11), and 45 h (lanes 3, 6, 9, and 12).

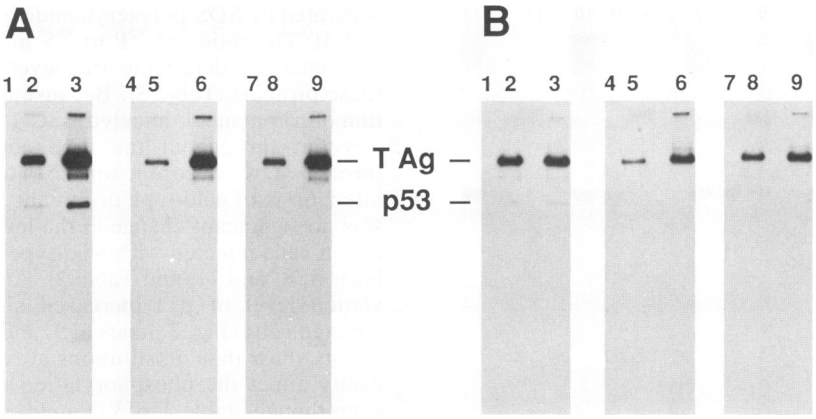

FIG. 4. Phosphorylation of T antigen during productive infection of BSC-1 cells. Subconfluent BSC-1 cells were infected with wild-type SV40 or mutant 402DN or 402DH. The cells were labeled with [^{32}P]phosphate (A) or L-[^{35}S]methionine (B) for 3 h. Cells were lysed, and the lysates were incubated with anti-T monoclonal antibody pAb416. Labeled proteins were analyzed as described in the legend to Fig. 3. Lanes 1 through 3, Wild-type SV40; lanes 4 through 6, 402DN; lanes 7 through 9, 402DH. Cells were harvested at 0 h (lanes 1, 4, and 7), 20 h (lanes 2, 5, and 8), and 45 h (lanes 3, 6, and 9).

virus replication in the monkey host. Either of these two scenarios would explain why T antigen's ability to bind to p53 has been retained during evolution.

Mutations at T-antigen residue 402 also affected binding to mouse p53, although they did not totally abolish binding. The conservative substitution of Asp-402 with Glu had a slight effect on binding to mouse p53, whereas more severe substitutions to Asn or His had greater effects (Fig. 2, lanes 8 through 13, and Table 2). No other mutation of residues 388 to 411 negatively affected the binding to p53, with the exception of a change at residue 401 (Table 1 and Fig. 1, lanes 4 and 5). Hence, within this region, residues 401 and 402 are important for binding, with Asp-402 playing a much more dominant role. It is reasonable to hypothesize that Asp-402 is a contact residue for p53 binding.

TABLE 3. Phosphorylation of T antigen and p53 in SV40-infected monkey BSC-1 cells

Virus or type of infection	Time p.i. (h)	^{32}P/^{35}S[a]		
		T antigen	p53	
			Expt 1	Expt 2
Mock infection	0		2.17	ND[b]
	20		1.63	ND
	45		1.16	ND
WT SV40[c]	0		1.15	1.70
	20	1.27	1.92	1.36
	45	2.21	9.18	6.77
402DE	0		ND	1.00
	20	1.54	ND	1.09
	45	2.43	ND	4.14
402DN	0		1.81	ND
	20	1.95	1.36	ND
	45	2.17	5.33	ND
402DH	0		1.72	ND
	20	1.05	1.04	ND
	45	2.40	12.40	ND

[a] Ratio of ^{32}P-labeled T antigen to ^{35}S-labeled T antigen or ^{32}P-labeled p53 to ^{35}S-labeled p53.
[b] ND, Not done.
[c] WT, Wild type.

The observed differences in the binding of mutant T antigens to primate (human and monkey) p53 and mouse p53 suggest that Asp-402 is only one of several contact sites for binding. It has been reported that T antigen forms more stable complexes with rodent p53 than with primate p53 (19). We suggest that this is due to a greater number of binding sites for rodent p53. The mutation of one of the sites involved in the binding to all p53s would affect the weaker interaction with primate p53 more than the stronger interaction with rodent p53.

T antigen also complexes with the product of the retinoblastoma susceptibility gene (Rb) in infected and transformed cells (8, 11). The binding region on T antigen has been mapped to residues 105 to 114 (6, 8). This tight clustering of contact residues in T antigen is unlikely to be present for binding to p53. Since only one or two sites between residues 388 and 411 are important for p53 binding, the other contact points are likely to map elsewhere in the protein. Tack et al. (46) showed that a mutant T antigen with a substitution at residue 584 (Pro to Leu) was unable to form stable complexes with p53. However, this mutant virus was defective in replication, and the mutant T antigen lost many of its biochemical activities (46). It seems likely that this mutation directly affected protein structure rather than an active site for p53 binding.

The results of our phosphorylation experiments indicate that a stable association between T antigen and p53 is not required for enhancing the phosphorylation of either protein during infection. It is known that phosphorylation functions to regulate T antigen's activities during productive infection (26, 29, 32, 37, 39, 54). However, it is not clear whether phosphorylation of p53 is also important for virus replication. T antigen, or possibly T-p53 complexes, induces or activates one or more protein kinases to phosphorylate p53 (34). One of these kinases may be the cdc2 kinase, since this protein associates weakly with p53 in vivo (28). Our data indicate that stable binding of T antigen to p53 is not important for this activation. It remains possible that a transient association between T antigen and p53 in infected cells is sufficient for kinase activation.

In summary, we have demonstrated that stable complexes of T antigen and p53 are not required for virus replication or increased phosphorylation of either protein in monkey cells. Increased phosphorylation may be due to the activation of a kinase (or kinases) by T antigen directly or by short-lived complexes between T antigen and p53. We have also shown that Asp-402 (and possibly Met-401) is important for binding to primate and rodent p53 proteins.

ACKNOWLEDGMENTS

We thank Jacques Pène for preparing the oligonucleotides and Huey-Jen Lin for help with the figures and tables.

This work was supported by grant CA36118 from the National Cancer Institute.

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