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Simian Virus 40 Can Overcome the Antiproliferative Effect of Wild-Type p53 in the Absence of Stable Large T Antigen-p53 Binding

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In simian virus 40 (SV40)-transformed cells, a tight complex is formed between the viral large T antigen (large T) and p53. It has been proposed that this complex interferes with the antiproliferative activity of p53. This notion was tested in primary rat fibroblasts by assessing the ability of SV40-mediated transformation to be spared from the inhibitory effect of wild-type (wt) p53. The data indicate that relative to transformation induced by *myc* plus *ras*, SV40-plus-*ras*-mediated focus formation was indeed much less suppressed by p53 plasmids. A majority of the resultant cell lines made a p53 protein with properties characteristic of a wt conformation. Furthermore, cell lines expressing stably both SV40 large T and a temperature-sensitive p53 mutant continued to proliferate at a temperature at which this p53 assumes wt-like properties and normally causes a growth arrest. Surprisingly, at least partial resistance to the growth-inhibitory effect of wt p53 was also evident when transformation was mediated by an SV40 deletion mutant, encoding a large T which does not bind p53 detectably. In addition to supporting the idea that SV40 can overcome the growth-restrictive activity of wt p53, these findings strongly suggest that at least part of this effect does not require a stable association between p53 and large T.

The simian virus 40 (SV40) large T antigen (large T) is capable of immortalizing and transforming a large variety of cell types (63). The cellular events underlying these oncogenic activities of large T are still poorly understood. However, the specific physical association between large T and proteins such as p53, Rb, and the 107-kDa polypeptide (4, 7, 27, 31) are believed to play a key role in this process. Unlike their wild-type (wt) counterparts, tumor-derived mutants of both p53 and Rb often fail to interact with large T or exhibit a markedly reduced association with this viral protein (18, 22, 24, 56, 61). Both wt p53 and Rb have been shown to possess antiproliferative activities (1, 3, 23, 39, 42), and it therefore appears most likely that the physical interaction with large T interferes with these activities and thereby contributes to neoplastic cell properties (4, 17, 28).

In SV40-transformed cells, the formation of the large T-p53 complex is correlated with a dramatic increase in the stability of p53, resulting in the accumulation of very high levels of this protein (46, 47). While this could imply that the mere formation of the complex protects p53 from proteolysis, more recent data suggest that this may not be the case. Hence, in some SV40-transformed cells, free p53 is almost as stable as large T-bound proteins (8). Moreover, in abortively infected mouse fibroblasts, one can find large T-bound p53 which is nevertheless short-lived (9). In addition, while certain mutations increase the stability of p53, this stabilization also depends on a particular intracellular environment, and most probably on the state of transformation of the cell (19, 25, 51). All of these observations imply that the increased metabolic stability of p53 in SV40-transformed cells may be mediated through an effect of SV40 on cellular properties rather than, or in addition to, complex formation per se. By inference, if the high levels of stable p53 in SV40-transformed cells represent the accumulation of functionally impaired protein, then one could propose that the inactivation of p53's antiproliferative capacity is also dependent on SV40-induced cellular alterations.

We have previously shown that overproduction of a mutant p53 enhances transformation by wt large T and partially complements a transformation-defective large T mutant (40). A likely interpretation is that the mutant contributes to extinguishing an inhibitory activity of the endogenous wt p53 (11, 15, 17), thereby alleviating a rate-limiting step in SV40-mediated transformation. The particular p53 mutant used in those studies, $p53_{Val-135}$, has recently been shown to be temperature sensitive (*ts*). At 32.5°C, $p53_{Val-135}$ can exert wt-like activities; i.e., it can suppress oncogene-mediated transformation and reversibly block cell proliferation (42).

In this study, we investigated whether SV40 could indeed overcome the antiproliferative effects of wt p53. In addition, we examined the relevance of the large T-p53 complex for the putative inactivation of wt p53 by SV40. Our findings indicate that SV40-mediated transformation is relatively resistant to inhibition by wt p53, as is the proliferation of stable cell lines exposed to wt-like p53 through the use of the *ts* mutant. Furthermore, partial resistance can also be conferred when transformation is mediated by an SV40 mutant encoding a non-p53-binding large T. Hence, complex formation between p53 and large T is not an absolute prerequisite for the ability of SV40 to overcome the inhibitory activity of p53.

MATERIALS AND METHODS

Plasmids. Activated human Ha-*ras* is encoded by pEJ6.6 (57); pLTRmyc directs the synthesis of mouse c-*myc* (45). pSVBam and *dl*1001 carry wt SV40 and a deletion mutant thereof, encoding a truncated large T, respectively (40, 50). pCMVp53wt contains the human cytomegalovirus immediate-early enhancer-promoter linked to *wt* mouse p53 cDNA (11). pCMVp53m encodes $p53_{Gly-168,Ile-234}$ and is otherwise identical to pCMVp53wt (11). pCMVp53dl is a highly deleted derivative of pCMVp53wt that can potentially encode only

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the first 13 N-terminal amino acids of p53 (11). pLTRp53 cGwt, pLTRp53cGval135, and pLTRp53cGphe132 encode wt p53, $p53_{Val-135}$, and $p53_{Phe-132}$, respectively (42). pLTRp53dl is a deleted derivative of pLTRp53cGval135 (42). Plasmid pRSVTAg, encoding SV40 large T under the control of the Rous sarcoma virus long terminal repeat, was a kind gift of A. Rozenthal (Genentech, Inc.).

Cell culture and transfections. Low-passage primary rat embryo fibroblasts (REF) and all cell lines were maintained in Dulbecco's modified Eagle's medium containing 5 to 10% fetal calf serum. REF were prepared and transfected by the calcium phosphate coprecipitation method as described before (12). All cell lines described below originated in REF which were transformed by different oncogene combinations. Clone 6 cells are transformed by $p53_{Val-135}$ and *ras* (49). RMp53val135-2 cells were isolated following transfection of REF with pLTRp53cGval135, pLTRmyc, and pEJ6.6. SV-R-p53wt lines 1 to 4 were derived from REF transfected with pSVBam, pEJ6.6, and pCMVp53wt. In a second independent experiment, SVRCwp53 lines 1 to 4 were isolated following an identical transfection. SVRp53 val135-4 originated in a transfection with pSVBam, pEJ6.6, and pLTRp53cGval135. Cell lines 1001-R-p53wt 1 to 10 as well as 1001RCwp53-1 and -2 were isolated following transfection with *dl*1001, pEJ6.6, and pCMVp53wt. Cell lines 1001-R-p53m-1, -2, and -3 originated in a transfection with *dl*1001, pEJ6.6, and pCMVp53m. 1001Rp53phe132-3 and -4 were generated by transfection with *dl*1001, pEJ6.6, and pLTRp53cGphe132. 1001Rp53val135-3 and -4 were obtained by transfection with *dl*1001, pEJ6.6, and pLTRp53cGval135.

Transient transfection assays were performed by the calcium phosphate method as described before (11).

Immunoprecipitation and protein analysis. Cells were labeled with [^{35}S]methionine, and cell extracts were prepared as described previously (33). Aliquots containing equal amounts of trichloroacetic acid-insoluble radioactivity were subjected to immunoprecipitation with the anti-p53 monoclonal antibodies (MAbs) PAb421 (20) and PAb246 (64). Immunnoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5% gels (33). Gels were fluorographed with 2,4-diphenyloxazole–dimethyl sulfoxide, dried, and exposed to Agfa Curix X-ray film.

Steady-state levels of p53 were determined by Western immunoblotting. Nonradiolabeled cell extracts were prepared essentially as for radiolabeled samples. Aliquots of each extract, containing 50 μg of total protein, were separated by SDS-PAGE and transferred to nitrocellulose. The blot was incubated with MAb RA3-2C2 (52) (hybridoma culture supernatant, diluted 1:20 in phosphate-buffered saline containing 10% low-fat milk and 0.05% Tween 20). The blot was developed by using peroxidase-conjugated protein A in combination with the ECL chemiluminescence detection system (Amersham).

RESULTS

Transformation by SV40 is relatively refractory to the inhibitory effects of wt p53. The ability of various oncogene combinations to elicit transformed foci in transfected REF is greatly reduced in the presence of wt p53 expression plasmids (11, 14). This is true when transformation is mediated by either mutant p53 plus *ras*, *myc* plus *ras*, adenovirus E1a plus *ras*, or E1a plus E1b. If SV40 large T can indeed overcome the antiproliferative properties of wt p53, one would expect SV40-mediated transformation to be more resistant to the inclusion of wt p53 plasmids. In the series of experiments described below, REF were transfected with combinations consisting of an SV40 plasmid, a mutant *ras* plasmid, and a wt p53 plasmid. The inclusion of *ras* served three purposes. First, it increased the number and ease of identification of foci induced by wt SV40 (41). Second, it provided a better comparison with other oncogene combinations, which typically include *ras* as a common member. Finally, it allowed the use of deletion mutants of SV40, which transform REF only when introduced together with *ras* (41). In this study, we used SV40 mutant *dl*1001 (50), which encodes a truncated large T, comprising the N-terminal 272 residues of the viral protein. This large T fails to exhibit any detectable association with p53 (41) and can cooperate efficiently with *ras* in the REF cotransformation assay (41).

In the first set of experiments, REF were cotransfected with either wt SV40 or *dl*1001, together with *ras* and a plasmid encoding either wt p53 (pCMVp53wt) or a mutant (pCMVp53m) incapable of suppressing oncogene-mediated transformation (11, 18). Plasmid pCMVp53dl, lacking the majority of the p53 coding region and specifying only the first 13 amino acids, was included as a negative control and as a carrier to keep DNA inputs constant in each transfection dish.

Plasmid pCMVp53wt can efficiently suppress *myc*-plus-*ras*-mediated transformation (11). In agreement with our earlier data, the extent of suppression was better than 10-fold (Fig. 1A; Table 1). In contrast, SV40-mediated transformation was much less sensitive to similar amounts of pCMVp53wt. Compared with the control plasmids, pSP6 and pCMVp53dl, the wt p53 vector reproducibly caused only a mild reduction in focus number (Fig. 1A; Table 1; data not shown). Quite unexpectedly, transformation by *dl*1001, an SV40 mutant encoding a non-p53-binding large T, was also relatively refractory to pCMVp53wt, indicating that it too could overcome the growth-inhibitory capacity of wt p53. It is noteworthy that with both SV40 plasmids, increasing amounts of wt p53 DNA did not lead to a further reduction in focus number. Such behavior may suggest the existence, within the population of primary cells, of a subpopulation in which SV40 is not able to overcome the antiproliferative effect induced by pCMVp53wt, while the rest of the cells are essentially refractory to this effect when transformed by SV40.

To determine whether the observed resistance to inhibition by wt p53 depended on the nature of the expression vector encoding this protein, pCMVp53wt was substituted by pLTRp53cGwt. The latter is a very potent inhibitor of focus formation (42) and is essentially identical to the plasmid used by Finlay et al. (14). As can be seen in Fig. 1B, this plasmid was indeed more efficient in suppressing transformation by mutant *myc* plus *ras*, completely eliminating focus induction at relatively low DNA inputs. Yet even with this very inhibitory plasmid, SV40-dependent focus formation was markedly less sensitive than *myc*-plus-*ras*-mediated transformation.

Expression of transfected p53 in SV40-transformed cells. Following transfection of REF with *myc* plus *ras* plus pCMVp53wt, a few foci do arise. However, these foci usually do not express any transfected p53 (11). In the rare cases in which such a protein is expressed, it bears evidence of having undergone a mutation, presumably giving rise to the loss of the growth-inhibitory activity of p53 (39a). An essentially similar picture has been observed in REF trans-

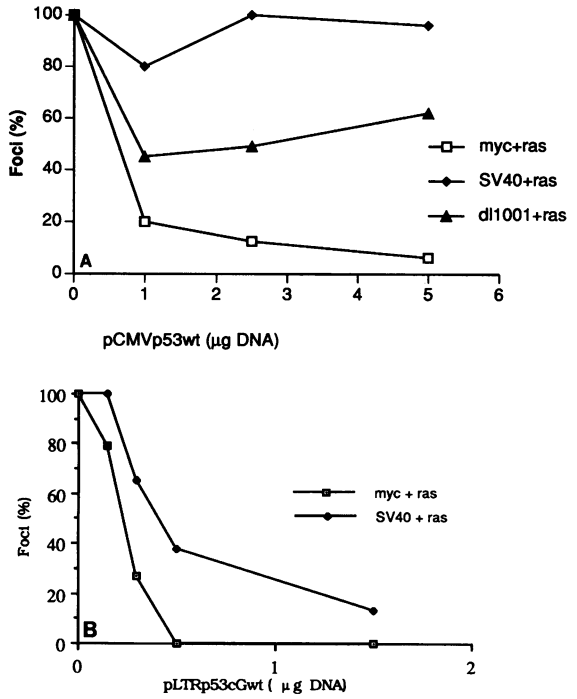

FIG. 1. Dose-dependent inhibition of oncogene-mediated transformation by plasmids encoding wt p53. REF were transfected with plasmids encoding *ras* and either *myc*, wt SV40, or *dl*1001 in the presence of variable amounts of the wt p53-encoding plasmid pCMVp53wt (A) or pLTRp53cGwt (B). Amounts of the oncogenic plasmids were as in Table 1. Amounts of DNA in each transfection were kept constant by the addition of pCMVp53dl to a total amount of 5 μg (A) or pLTRp53dl to a total of 1.5 μg (B). Foci were scored 11 days posttransfection.

fected by E1a plus *ras* in the presence of a wt p53 plasmid (14). If SV40 can, in fact, protect transformed cells from the antiproliferative activity of wt p53, one could expect to observe wt p53 expression in resultant cell lines. This should help distinguish the induction of tolerance to wt p53 from other, nonspecific mechanisms such as down-regulation of p53 expression or increased plasmid breakdown. To address this issue, cell lines were generated from foci induced by either wt SV40 plus *ras* or *dl*1001 plus *ras* in the presence of wt p53 plasmids. The results of such analysis are shown in Fig. 2. When mutant p53 ($p53_{Gly\text{-}168,Ile\text{-}234}$), encoded by plasmid pCMVp53m, was cotransfected with *dl*1001 and *ras*, abundant expression of mouse p53 could be seen in two of three lines tested (Fig. 2C, 1001-R-p53m). As expected, this mutant p53 formed an easily visible complex with the major heat shock protein cognate 70 (hsc70). A similar analysis was next performed on lines derived from REF transfected by *ras* and either wt SV40 or *dl*1001 in the presence of pCMVp53wt. Most of the lines did express exogenous mouse p53, both in the case of wt SV40 (Fig. 2A and B) and in the case of *dl*1001 (Fig. 2C and D). Figure 2A represents a short exposure of the same lanes as in Fig. 2B to allow a better resolution between the transfected (mouse) and endogenous (rat) p53. Of a total of 15 lines originating in cells transfected with *ras* plus wt SV40 plus pCMVp53wt, 13 expressed easily detectable amounts of mouse p53 (Fig. 2 and 3 and data not shown). Similarly, 16 of 19 lines derived from transfections in which *dl*1001 was used instead also expressed abundant mouse p53. In the case of transfection by *ras* plus *dl*1001 plus pLTRcGwt, four of six lines were positive for exogenous p53 (data not shown). In certain lines, the mouse p53 was associated to various extents with hsc70 (Fig. 2B, lanes 1 and 2). Overall, this was true for 5 of the 13 p53-positive lines generated by *ras* plus wt SV40 plus pCMVp53wt and 3 of the 16 positive lines generated by *ras* plus *dl*1001 plus pCMVp53wt (data not shown). Such an interaction is characteristic of mutant forms of p53 (15, 21, 49, 59) and implies that the transfected wt p53 gene must have been mutated at a certain point during or after the initial transforming events. In most cell lines, however, there was no evidence for an aberrant behavior of p53. This raised the possibility that, at least in some of these lines, the exogenous p53 remained wt despite the fact that the transformed cells could proliferate avidly. Unlike lines transfected with pCMVp53wt, those productively transfected with pLTR p53cGwt typically made p53 which formed a tight association with hsc70 (data not shown). Thus, in the case of this very inhibitory plasmid, it seems that the only p53-expressing cells that generate lines may be those in which the p53 gene has undergone mutations.

To obtain additional support for the suggestion that some pCMVp53wt-transfected lines expressed wt p53, the protein made in those lines was subjected to immunoprecipitation with MAb PAb246 (64). Reactivity with the latter is considered to be a good indication of a wt-like p53 conformation (15, 16). Figure 3 shows results of such an analysis performed with a set of cell lines different from those shown in Fig. 2. As can be seen, the vast majority of lines expressing pCMVp53wt-encoded p53 made a protein capable of reacting very efficiently with PAb246 compared with the reactivity with PAb421, which binds both mutant and wt p53 (15, 16). In addition, most lines in this series did not exhibit any binding to hsc70. The only exception was line SVRCwp53-3 (Fig. 3C), which seems to make a mixture of wt and mutant p53. These properties are in striking contrast to those exhibited by the lines shown in Fig. 3A, in which the transfected p53 plasmid encoded a mutant protein, $p53_{Phe\text{-}132}$. In these lines, there was a clear coprecipitation of hsc70 and hardly any reactivity with PAb246. Thus, by these

TABLE 1. Effects of wt and mutant p53 on SV40-, *dl*1001-, and *myc*-mediated focus formation[a]

Transfected plasmids	No. of foci/dish[b]		
	Expt 1	Expt 2	Expt 3
pEJ6.6 + pSVBam + pSP6	126	111	ND
pEJ6.6 + pSVBam + pCMVp53dl	137	ND	155
pEJ6.6 + pSVBam + pCMVp53m	123	ND	ND
pEJ6.6 + pSVBam + pCMVp53wt	75	40	87
pEJ6.6 + pdl1001 + pSP6	73		ND
pEJ6.6 + pdl1001 + pCMVp53dl	122		35
pEJ6.6 + pdl1001 + pCMVp53m	116		ND
pEJ6.6 + pdl1001 + pCMVp53wt	40		15
pEJ6.6 + pLTRmyc + pCMVp53dl			23
pEJ6.6 + pLTRmyc + pCMVp53wt			2

[a] REF were transfected with expression plasmids specific for *ras* (pEJ6.6, 2.5 μg per dish), wt SV40 (pSVBam, 0.5 μg per dish), the *dl*1001 deletion mutant of SV40 (1.5 μg per dish), and *myc* (1.5 μg per dish). Different oncogenic DNA mixtures were supplemented with one of the plasmids encoding mutant or wt p53 or with one of the control plasmids: pSP6 or pCMVp53dl (5 μg per dish). For further details about these plasmids, see Materials and Methods. Foci were scored 10 to 14 days posttransfection.

[b] Values represent averages of the number of foci in two parallel dishes. ND, not done.

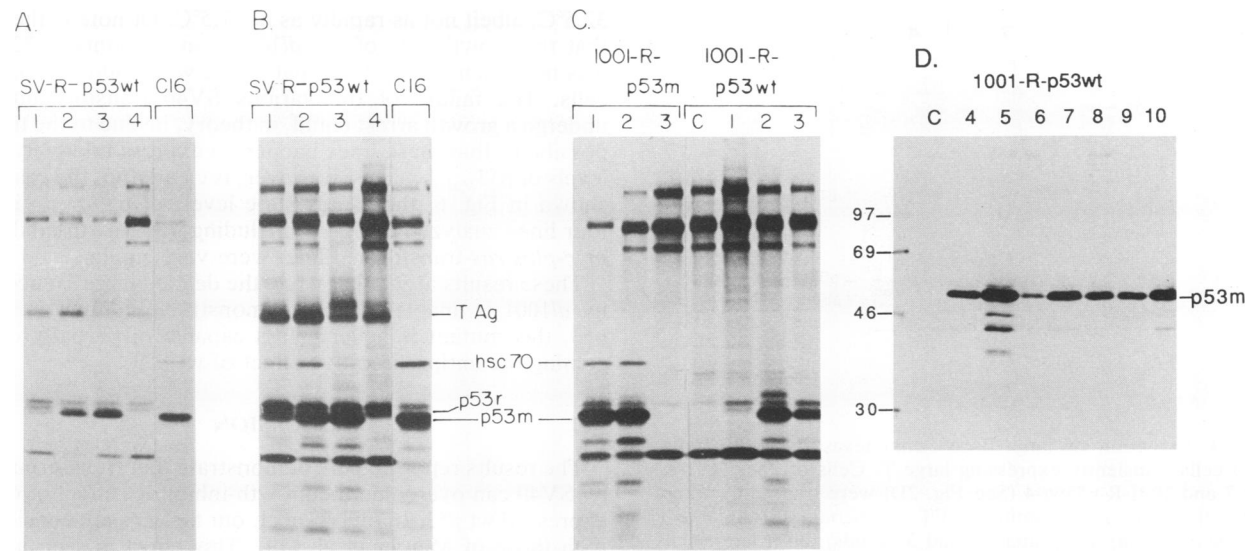

FIG. 2. Analysis of p53 expression in transformed cell lines. REF were transfected with plasmids encoding *ras*, wt SV40, and wt p53 (pCMVp53wt) or with *ras*, *dl*1001, and wt or mutant p53 (pCMVp53wt or pCMVp53m). DNA inputs per dish were identical to those described in Table 1. Transformed foci were isolated and expanded into cell lines. Cells were labeled with [^{35}S]methionine for 3.5 h, extracts were made, and p53 was immunoprecipitated with MAb PAb421 and subjected to SDS-PAGE. Each lane of the autoradiogram represents an immunoprecipitate from one cell line. (A and B) SV-R-p53wt, cell lines derived from foci induced by a combination of *ras*, wt SV40, and pCMVp53wt. A and B represent different autoradiographic exposures of the same gel (see text). (C) 1001-R-p53m, cell lines derived from foci induced by a combination of *ras*, *dl*1001, and pCMVp53m. 1001-R-p53wt cells are derived from foci induced by *ras*, *dl*1001, and pCMVp53wt. Lane C, extract of line 1001-R-p53wt-2 reacted with control nonimmune hybridoma culture medium. (D) 1001-R-p53wt, cell lines derived from an independent transfection with *ras*, *dl*1001, and pCMVp53wt. Positions and molecular masses (in kilodaltons) of marker polypeptides are indicated on the left. Cl6, clone 6, derived from REF transformed by *ras* plus $p53_{Val-135}$; p53r, endogenous rat p53; p53m, transfected mouse p53; T Ag, T antigen.

criteria, the p53 protein made in most pCMVp53wt-transfected lines appears to be wt.

Another characteristic property of wt p53 is its ability to bind SV40 large T. The loss of this efficient binding to large T is the only property which is common to all non inhibitory p53 mutants analyzed thus far (5, 18, 24, 44, 61), raising the possibility that it is essential for the antiproliferative effect of wt p53 (43). To further establish the wt nature of the p53

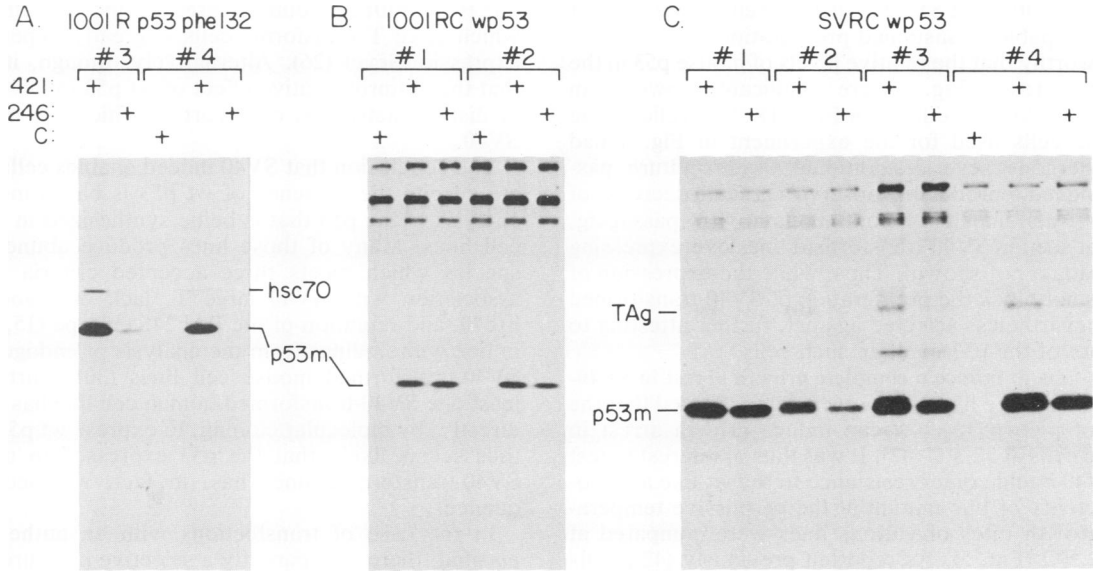

FIG. 3. Immunological characterization of p53 in transformed cell lines. REF were transfected with plasmids encoding *ras* and either wt SV40 (C) or *dl*1001 (A and B) in the presence of pLTRp53cGphe132 (A) or pCMVp53wt (B and C). Cell lines were isolated and labeled with [^{35}S]methionine for 3.5 h. Extracts were subjected to immunoprecipitation with MAb PAb421 or PAb246 or with control hybridoma culture medium (C). Numbers denote individual cell lines.

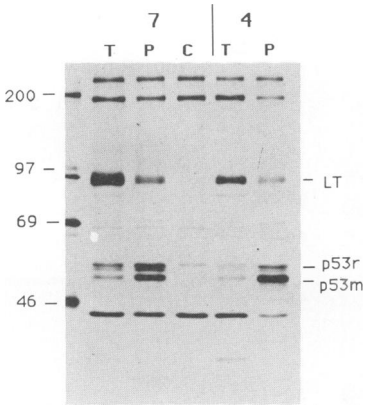

FIG. 4. Detection of large T-p53 complexes in *dl*1001-transformed cells transiently expressing large T. Cells of lines 1001-R-p53wt-7 and 1001-R-p53wt-4 (See Fig. 2D) were transiently transfected with expression plasmid pRSVTAg, encoding SV40 large T (30 μg/90-mm dish; see Materials and Methods); 36 h later, cells were labeled for 5 h with [^{35}S]methionine and subjected to immunoprecipitation with either MAb PAb419, specific for large T (lanes T), MAb PAb421, specific for p53 (lanes P), or control hybridoma culture medium (C). Symbols are as in Fig. 2.

produced in the transformed lines, two such lines, derived from foci induced with pCMVp53wt plus *ras* plus *dl*1001, were transiently transfected with a plasmid encoding SV40 large T. Radiolabeled cell extracts were prepared and subjected to immunoprecipitation with MAbs specific for either p53 or large T. Clearly, the transfection-derived mouse p53 could indeed form stable interactions with large T, as demonstrated by the ability of the anti-large T MAb PAb419 to coprecipitate the mouse polypeptide along with the endogenous rat p53 (Fig. 4). Such tight complexes fail to be formed when large T is coexpressed with mutant mouse p53 (18). All of these data support the conclusion that many of the transfected cell lines indeed overproduce wt p53. Hence, in the presence of either wt SV40 or *dl*1001, cells expressing wt p53 are still capable of sustained proliferation.

It is noteworthy that the relative levels of mouse p53 in the two lines depicted in Fig. 4 were significantly lower than those seen with the same lines in Fig. 2D. This reflects the fact that the cells used for the experiment in Fig. 4 had already undergone several additional tissue culture passages. We indeed reproducibly observe a gradual decrease of p53 levels in such lines upon continuous in vitro passaging, a feature not seen in SV40-transformed lines overexpressing mutant p53 (data not shown). Thus, while the expression of wt p53 does not block the proliferation of SV40-transformed cells, it is nevertheless selected against, further attesting to the wt nature of the p53 made in such cells.

$p53_{Val-135}$ fails to induce a complete growth arrest in SV40-transformed cells. We have previously demonstrated that the *ts* mutant of p53, $p53_{Val-135}$, can induce growth arrest in transformed cells at 32.5°C (42). It was thus of interest to test whether SV40 could confer resistance to the wt-like antiproliferative activity of this mutant at the permissive temperature. The growth rates of various lines were compared at 37.5 and 32.5°C (Fig. 5). As reported previously (42), cells transformed by *myc* plus *ras*, which overexpress $p53_{Val-135}$, are very efficiently growth arrested at 32.5°C (line RMp53val135-2). On the other hand, cells transformed by *ras* and either wt SV40 or *dl*1001 continue to proliferate at 32.5°C, albeit not as rapidly as at 37.5°C. Of note is the fact that the growth rate of the *dl*1001 transformants at 32.5°C was not much slower than that of the wt SV40-transformed cells. The failure of the various SV40 transformants to undergo a growth arrest could, in theory, be due to the trivial possibility that these lines happen to express relatively low levels of $p53_{Val-135}$. This, however, is clearly not the case; as shown in Fig. 6, the steady-state levels of $p53_{Val-135}$ in all four lines analyzed in Fig. 5, including the growth-inhibited *myc-plus-ras*-transformed line, were very similar.

These results argue that while the deleted large T encoded by *dl*1001 is unable to form demonstrable complexes with p53, this mutant is nevertheless capable of partially overcoming the antiproliferative effect of wt p53.

DISCUSSION

The results reported here demonstrate that transformation by SV40 can overcome the growth-inhibitory effect of overexpressed wt p53. In this respect, our findings are consistent with those of Mercer et al. (39). This effect is seen at the levels of both initiation and maintenance of transformation. Thus, whereas the number of *myc-plus-ras*-induced REF foci is dramatically reduced in the presence of cotransfected wt p53 plasmids, the same plasmids cause at most a two- to three-fold reduction when transformation is driven by SV40 plus *ras*. A similar situation is manifested in stably transformed, continuously growing cell lines, which carry the *ts* mutant $p53_{Val-135}$. In *myc* plus *ras*-transformed lines, the overexpressed *ts* p53 causes a nearly complete growth arrest at 32.5°C, a temperature at which it exhibits features characteristic of wt p53 (42). On the other hand, the proliferation of cell lines transformed by SV40 plus *ras* is only partially impeded by the wt-like activity of $p53_{Val-135}$ at 32.5°C. Interestingly, overproduced human wt p53 was shown to interfere with the proliferation of SV40-transformed hamster cells (38). In this case, too, it was suggested that such transformants could tolerate a certain level of wt p53 expression (38). The fact that wt p53 still exerts a partial effect on both focus formation and cell proliferation could simply be due to a limiting amount of large T, since the efficiency with which large T transforms cells is greatly dependent on its expression level (26). Alternatively, though, it is possible that the antiproliferative effect of wt p53 involves a number of distinct activities, only part of which are overcome by SV40.

The conclusion that SV40 indeed enables cells to proliferate despite the presence of wt p53 is based in part on the analysis of the p53 that is being synthesized in transformed cell lines. Many of those lines produce abundantly a p53 species which meets three accepted criteria for wt p53: association with SV40 large T, lack of association with hsc70, and retention of the PAb246 epitope (15, 16). This is in line with findings from the analysis of endogenous p53 in SV40-transformed mouse cell lines (30). Furthermore, at least one SV40-transformed human cell line has been shown directly, by molecular cloning, to express wt p53 (35, 65). It thus seems likely that the p53 expressed in many of our SV40-transformed lines has, in fact, retained its wt sequence.

In the case of transfections with an authentic wt p53 plasmid, there is apparently a selective pressure against the antiproliferative effect of this protein, which may result in mutational inactivation of the incoming DNA. This argument does not hold for the lines derived in the presence of cotransfected $p53_{Val-135}$. This mutant, when expressed at

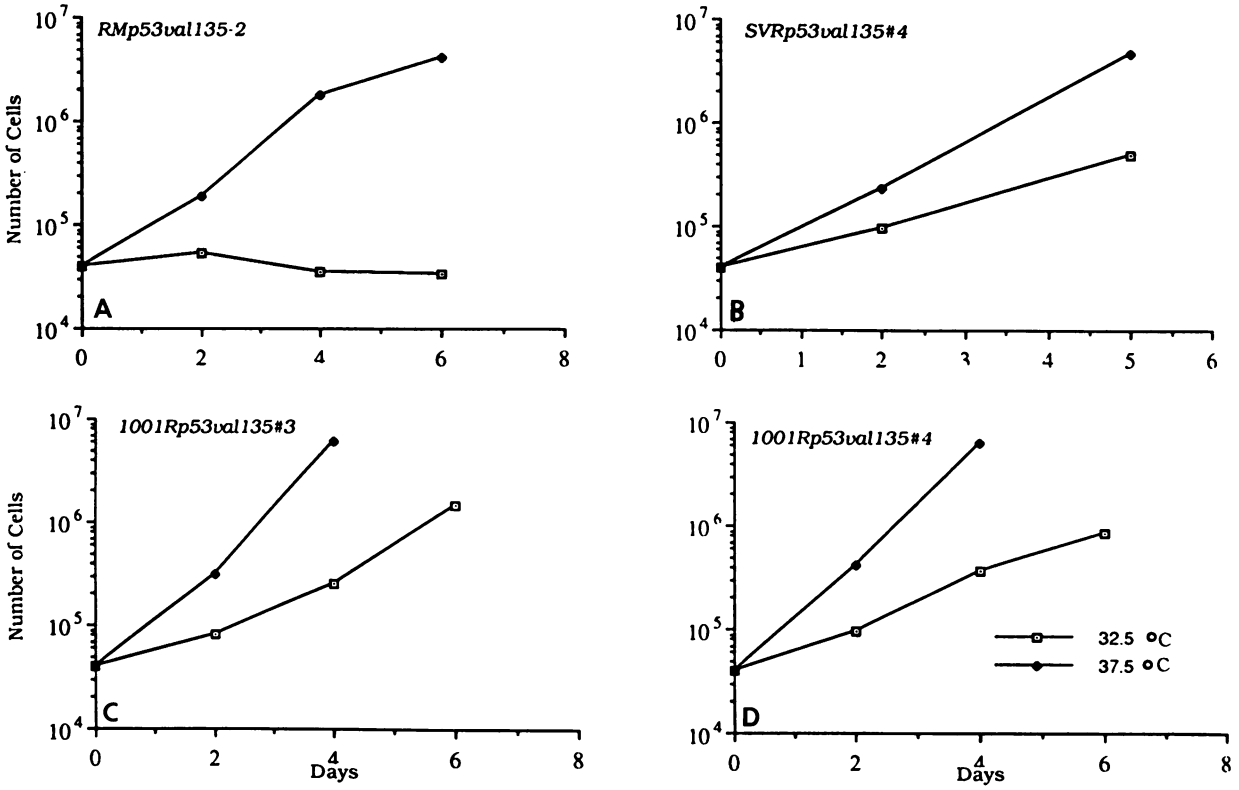

FIG. 5. Growth analysis of $p53_{Val-135}$-overexpressing lines at 37.5 and 32.5°C. Cells growing at 37.5°C were plated at a density of 40,000/60-mm dish and maintained at either 37.5 or 32.5°C for several days. At the indicated time points, cells were trypsinized and counted. (A) REF transformed by *ras*, *myc*, and $p53_{Val-135}$; (B) REF transformed by *ras*, wt SV40, and $p53_{Val-135}$; (C and D) two individual cell lines derived from a transfection with *ras*, *dl*1001, and $p53_{Val-135}$.

37.5°C, not only is not selected against but actually enhances transformation by both wt SV40 and *dl*1001 (40; data not shown). It is therefore reasonable to assume that the transfected p53 plasmids have retained their original sequence. Yet when shifted to 32.5°C, $p53_{Val-135}$ does not cause a complete block of proliferation, as it does efficiently in *myc*-plus-*ras* transformants. These results further argue that SV40-transformed cells possess enhanced tolerance to the growth-inhibitory signals generated by wt p53.

Perhaps the most surprising finding in this study is the ability of *dl*1001 to generate transformed cell lines that are at least partially refractory to the inhibitory effect of wt p53. The large T encoded by *dl*1001 contains only the first 272 residues of the authentic protein (50), whereas the p53-binding domain of large T has recently been mapped to a region located between residues 337 and 517 (34). This is reflected in the inability of the *dl*1001 large T to form stable complexes with p53, as reported earlier (41). Our results would therefore argue that SV40 can overcome the antiproliferative effect of wt p53 in the absence of complex formation between the latter and large T. This possibility is unexpected, since it is commonly assumed that the binding of p53 to large T sequesters the former in a functionally inactive form and thus eliminates its antiproliferative functions (17, 28, 29). The results presented above still do not rule out the possibility that direct binding to large T does contribute to p53 inactivation. In fact, at least in the focus formation studies, wt SV40 does confer a higher degree of resistance to wt p53 than does *dl*1001, as is evident from the dose-response analysis (Fig. 1A). However, we have observed that the levels of the truncated T made by *dl*1001-transformed cells are markedly lower than those of intact large T in cells transformed by wt SV40 (data not shown). Hence, the lower resistance of *dl*1001-mediated transformation to wt p53 may simply reflect the presence of suboptimal amounts of the corresponding viral protein.

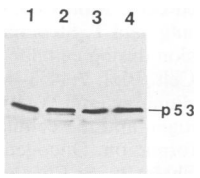

FIG. 6. Comparison of steady-state levels of p53 in cells overexpressing $p53_{Val-135}$. Nonradioactive extracts were prepared from cells of the four lines described in the legend to Fig. 5 and subjected to analysis by Western blotting as detailed in Materials and Methods. Lanes: 1, RMp53val135-2; 2, SVRp53val135-4; 3 and 4, 1001-R-p53val135-3 and -4, respectively.

The ability of *dl*1001 to confer resistance to p53-mediated growth inhibition suggests that at least some of the activities responsible for this property may reside in the N-terminal domain of the SV40 early region. A provocative possibility is that the protective effect is mediated through the ability of large T to interact with cellular proteins such as the Rb protein and the 107-kDa polypeptide (4, 7, 13, 32). However, this possibility is not supported by earlier experiments, in

which focus induction by adenovirus E1a plus *ras* was found to be quite sensitive to suppression by wt p53 (11, 14). Furthermore, transformation by *ras* plus the SV40 mutant 3213, carrying a defective Rb binding site (53), is highly refractory to suppression by wt p53 (16a).

One mechanism through which SV40 may potentially exert its protective effects is by affecting directly the molecular properties of the p53 protein. This could, for instance, involve covalent modifications that cause the cellular polypeptide to lose its growth-inhibitory function. In this respect, the most obvious candidate is a mechanism involving differential phosphorylation of p53. SV40-dependent alterations in the phosphorylation pattern of p53 have been reported by several groups (36, 54, 55), although this issue is still controversial (48). These alterations may be elicited by a protein kinase activity closely associated with large T (55). Interestingly, one of the sites implicated as differentially phosphorylated in SV40-transformed cells, serine 312 of mouse p53 (and serine 315 in human p53) (37), may be an in vivo target of the $p34^{cdc2}$ protein kinase (2, 60).

Another line of evidence also suggets that the mere formation of a complex between large T and p53 cannot account for all of the effects of SV40 on the latter. Stabilization of p53 by SV40 large T correlates well with the existence of physical associations between the two (46, 47). Taken together with the fact that mutants of p53, devoid of antiproliferative activity, are also usually much more stable (15, 19), this could imply that complex formation between p53 and large T renders the cellular protein inactive and concomitantly makes it very stable. However, work by Deppert and coworkers (8, 9) indicates that in nontransformed, abortively infected cells, p53 is labile even when bound to large T. On the other hand, in stably transformed cells, even unbound p53 is degraded much more slowly. In addition, studies with cells transformed by a *ts* mutant of SV40 large T revealed that in such cells, the stability of p53 correlated with the state of transformation rather than with the ability of large T to form complexes with p53 (10). These observations, too, could imply that the inactivation of p53 (as reflected by its stabilization) requires a more profound effect of SV40 rather than a simple association between p53 and large T.

Alternatively, the ability of *dl*1001-transformed cells to proliferate in the presence of wt p53 may not involve a direct effect on the p53 molecule itself. It is conceivable that the p53 made in such cells still remains functional, yet the cells are not severely affected by it. The underlying mechanism may, for instance, entail an alteration of a potential molecular target for wt p53 or some other sort of interference with p53-related growth-inhibitory signal transduction pathways.

The data reported above are reminiscent of recent additional findings concerning the biological activities of the N-terminal domain of SV40 large T. This region, retained in *dl*1001, is capable of immortalizing primary rodent cells (6, 58). It can bind stably another putative antiproliferative protein, the Rb gene product, and there is good evidence that this binding is required for the full oncogenic activity of large T (32). Nevertheless, it now appears that the actual Rb binding site, comprising residues 105 to 114 of large T (7, 13), is not required for efficient immortalization of primary cells and for tumorigenicity (62). Thus, another activity of SV40 may be responsible for these functions. A role for such additional activity is also supported by the demonstration that the combined effects of Rb binding and p53 binding are insufficient to account for the transforming potential of large T (34). It is tempting to speculate that the same activity also participates in conferring tolerance to wt p53.

Finally, if the binding of p53 to large T can indeed lead to the biochemical inactivation of the former, our findings would imply that SV40 has evolved multiple mechanisms for circumventing the restrictive effects of wt p53.

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ADDENDUM IN PROOF

It has recently been suggested that SV40 small t may modulate the activity of p53 (K. H. Scheidtmann, M. C. Mumby, K. Rundell, and G. Walter, Mol. Cell. Biol. **11:** 1996–2003, 1991). The complex-independent effect of SV40 on p53 may thus be exerted either by the N-terminal domain of large T or by the small t which is also encoded by the SV40 plasmids employed in our study.

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