Stabilization of the Tumor Suppressor p53 during Cellular Transformation by Simian Virus 40: Influence of Viral and Cellular Factors and Biological Consequences

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To understand the process and biological significance of metabolic stabilization of p53 during simian virus 40 (SV40)-induced cellular transformation, we analyzed cellular and viral parameters involved in this process. We demonstrate that neither large T expression as such nor the cellular phenotype (normal versus transformed) markedly influence the stability of p53 complexed to large T in SV40 abortively infected BALB/c mouse fibroblasts. In contrast, metabolic stabilization of p53 is an active cellular event, specifically induced by SV40. The ability of SV40 to induce a cellular response leading to stabilization of p53 complexed to large T is independent from the cellular phenotype and greatly varies between different cells. However, metabolic stability was conferred only to p53 in complex with large T, whereas the free p53 in these cells remained metabolically unstable. Comparative analyses of cellular transformation in various cells differing in stability of p53 complexed to large T upon abortive infection with SV40 revealed a strong correlation between the ability of SV40 to induce metabolic stabilization and its transformation efficiency. Our data suggest that metabolic stabilization and the ensuing enhanced levels of p53 are important for initiation and/or maintenance of SV40 transformation.

The transforming proteins of several DNA tumor viruses form tight complexes with the products of the tumor suppressor genes RB and p53 (9, 18, 41–43, 49, 66, 80, 81). Although the functional consequences of these interactions for cellular transformation are only partially understood at the molecular level, mutational analyses suggested that they are important for mediating the transforming functions of these proteins (7, 9, 38, 57, 81, 82). Best analyzed are the interactions of such proteins with pRB. Binding of pRB occurs via a pRB binding site, which is conserved among all viral pRB-binding proteins and fits into a structural domain (the pRB pocket) of pRB (32, 34, 36, 37). Binding of these proteins to pRB results in the release of the transcription factor E2F from a complex with pRB, in which E2F is in an inactive state (3, 5, 6, 33, 52, 54, 86). In contrast, no such general scheme regarding the interactions of viral transforming proteins with p53 has evolved, as these interactions differ between the transforming proteins of the various DNA tumor virus proteins physically as well as in their functional consequences. First, transforming proteins of different viruses interact with different domains on p53 (35, 59, 67, 74, 77), which suggests that their binding will affect p53 functions differently. This view is exemplified by comparing the effects of p53 binding by the E6 protein of human papovavirus types 16 and 18 with the binding of p53 by the transforming protein of simian virus 40 (SV40), the large tumor antigen (large T). Binding of p53 by the human papillomavirus E6 protein rapidly destines p53 to degradation by the ubiquitin-dependent proteolytic pathway, thus leading to an efficient functional elimination of p53 (68). On the other hand, p53 complexed to large T in SV40-transformed cells becomes

metabolically stabilized and accumulates to high levels (14, 39, 41, 49, 64). The molecular mechanism(s) underlying metabolic stabilization of p53 as well as the functional consequences of the ensuing accumulation of p53 to high levels still are controversial. Early experiments by Linzer et al. (50) showed that p53 complexed to large T in SV40 abortively infected 3T3 cells became metabolically stabilized, whereas p53 in the same cells, infected with a *tsA* mutant of SV40 at the nonpermissive growth temperature, was not complexed to the mutant large T and remained unstable. From these experiments, it was concluded that complex formation with large T as such induced metabolic stabilization of p53 (8, 50). This view was contrasted by experiments from our laboratory, which provided evidence that (i) SV40-transformed cells, in addition to p53 in complex with large T, harbor p53 which is not complexed to large T (free p53) but which nevertheless is metabolically stable (13) and that (ii) abortive infection of BALB/c 3T3 cells with SV40 did not result in a significant stabilization of p53 complexed to large T, while p53 in SV40-transformed cells arising from such abortively infected cells was metabolically stable (14). These data suggested that metabolic stabilization of p53 during SV40-mediated cellular transformation not simply was the result of its physical interaction with large T but rather was related to the establishment and/or maintenance of cellular transformation (10, 15).

Wild-type p53 is a tumor suppressor and is thought to be a negative regulator of cell growth (40; 44–47, 59). Mutations in the p53 gene are the most common genetic changes in a single gene in human tumors found to date (4, 31, 47). Most of these mutations are missense point mutations, which lead to the expression of a mutant p53 protein which has lost its tumor suppressor activity (2, 47, 55, 76). However, at least some mutations confer an overt oncogenic potential to the resulting mutant p53 (11, 16, 47, 83). Binding of large T to p53 eliminates wild-type p53-specific functions such as DNA binding and transactivation (20, 58). In this regard, wild-type p53 in

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complex with large T exhibits properties characteristic of a mutant p53 (44, 46, 59). If one assumes that complex formation of p53 with large T results in a mere elimination of p53 function and that its metabolic stabilization in SV40-transformed cells simply correlates with the cells acquiring a transformed phenotype (30, 46), binding of p53 by large T would be functionally equivalent to human papillomavirus E6-mediated degradation of p53. Alternatively, the metabolically stable wild-type p53 in complex with large T, like mutant p53, might have not only lost its tumor suppressor activity but also acquired dominant oncogenic properties. Then metabolic stabilization of p53 during SV40-mediated cellular transformation would reflect an active event in SV40-mediated cellular transformation, with the enhanced levels of p53 being advantageous for the establishment and/or maintenance of transformation; i.e., the stabilized p53 would act as a cooperating oncogene in SV40 transformation, as suggested previously (8, 10, 15).

To further define the role of metabolic stabilization of p53 in SV40-mediated cellular transformation, in this study we analyzed viral and cellular parameters leading to metabolic stabilization of p53 during abortive infection of mouse and rat fibroblasts with SV40. In particular, we wanted to determine the possible influences of the cellular phenotype (normal versus transformed) on the metabolic stabilization of p53 complexed to large T. Our data demonstrate that metabolic stabilization of p53 during abortive infection correlates neither with complex formation with large T nor with the cellular phenotype as such. In contrast, we provide evidence that metabolic stabilization of p53 reflects a specific and active cellular process, which is induced by SV40. Depending on the cell type, SV40 is able to induce this cellular process during abortive infection or only during establishment of transformation. Metabolic stabilization of p53 during abortive infection correlates with a strongly enhanced transformation efficiency, further supporting the idea that metabolic stabilization of p53 and the ensuing enhanced levels of p53 constitute an important step in SV40 mediated cellular transformation.

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MATERIALS AND METHODS

Cells. Primary mouse embryo fibroblast (MEF) cultures were prepared from mechanically dispersed 17-day-old BALB/c mouse embryos by digestion with 0.2% collagenase (type 1; Biochrom KG, Berlin) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Primary MEF cultures were grown in DMEM containing 5% newborn calf serum and frozen in liquid nitrogen after one passage. Precrisis MEF day 17 (pMEFd17) cultures were derived from primary cells. Thawed primary cells (10^5) were plated into 100-mm-diameter culture dishes in DMEM supplemented with 5% newborn calf serum and passaged according to the 3T3 schedule established by Todaro and Green (75). After an initial period of rapid growth lasting six to seven transfers, the growth rate of the pMEFd17 culture declined. Therefore, pMEFd17 cells were used for experiments between passages 3 and 5. BALB/c 3T3 cells (1) and the SV40-transformed BALB/c 3T3 cell line SV3T3 clone 4 (cl.4) (14) were grown as described previously (13). Normal Fischer rat fibroblast F111 cells (21) and Fischer rat fibroblast FR3T3 cells (23, 60, 61) were grown in DMEM supplemented with 10% FCS.

Progression of normal BALB/c 3T3 fibroblasts. Following the schedule for establishing BALB/c 3T12 cells (1), BALB/c 3T3 cells were continually maintained at high cell density.

Every 3 days, cells were transferred to fresh medium in new 60-mm-diameter culture dishes at 12×10^5 cells per dish. Two independently carried mass cultures, BALB/c 3T3-4 and BALB/c 3T3-10, were generated after periods of 4 and 10 weeks, respectively. Clonal lines BALB/c 3T3-cl4 and BALB/c 3T3-cl10 were obtained from these mass cultures by single-cell cloning.

Abortive infection. Normal mouse and rat fibroblasts were infected with wild-type SV40 (strain 776) at a multiplicity of infection (MOI) of 1, as described below, and analyzed 24 and 48 h postinfection (p.i.). The MOI for abortive infections was determined by infecting cells with SV40 diluted with DMEM plus 5% normal calf serum in consecutive 1:10 dilution steps. An MOI of 1 in abortive infections was defined as the dilution of SV40 at which more than 90% of the infected cells were positive for SV40 large T expression, as judged by immunofluorescence microscopy analysis. It is important to note that the MOI determined this way does not correspond to the MOI of the same virus stock in permissive monkey TC7 cells. Furthermore, an MOI of 1, as defined here, was cell specific and required individual titration of the virus stock for each cellular system analyzed.

Cloning in soft agar. Cells were plated in four parallel assays at totals of 10^5 , 10^4 , and 10^3 per 35-mm-diameter culture dish in DMEM containing 10% FCS and 0.3% (wt/vol) agar (Difco Bacto Agar; Difco Laboratories) onto a bottom layer of 0.5% (wt/vol) agar in DMEM. Colonies were scored 14 days after plating and designated positive when larger than 20 cells. Average values are presented.

Pulse-chase experiments. Equal numbers of subconfluent cells were pulse-labeled (15- to 60-min pulse; see figure legends) and pulse-chase-labeled (15- to 60-min pulse and four different periods of chase [c_1 , c_2 , c_3 , and c_4 ; see figure legends]) with [^{35} S]methionine/cysteine (Translabel; ICN) in 2 ml of methionine- and cysteine-free DMEM containing 5% dialyzed FCS per 100-mm-diameter dish.

Lysis, antibodies, immunoprecipitation, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting. Cells were washed twice with ice-cold phosphate-buffered saline and extracted for 30 min at 4°C with lysis buffer [50 mM Tris-HCl, 120 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM dithiothreitol, 1 mM ethylene glycol bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid] (EGTA), 30 μ g of aprotinin per ml (200 kIU; Trasylol; Bayer), 50 μ M leupeptin (pH 8.0)]. Extracts were cleared by centrifugation for 30 min at $12,000 \times g$ and 4°C.

SV40 large T and SV40 large T-p53 complexes were immunoprecipitated with monoclonal antibodies PAb108 (25) and PAb419 (27). p53 was immunoprecipitated with monoclonal antibodies PAb122 (24), PAb421 (27), PAb246 (84), and PAb240 (22). Immunoprecipitation and analysis by SDS-PAGE were carried out as described previously (72, 79). Labeled proteins were visualized by fluorography. SV40 large T and p53 were quantitated by densitometric scanning and represented in a logarithmic diagram. Western blot (immunoblot) analysis of steady-state levels of p53 and large T was performed as described previously (70, 85).

RESULTS

Metabolic stabilities of p53 in uninfected, SV40-infected, and SV40 stably transformed BALB/c 3T3 cells. BALB/c 3T3 cells are established mouse fibroblasts displaying a normal phenotype and growing to a low saturation density (1) (Table 1). These cells express low levels of a phenotypic (48, 73) and genotypic (unpublished data) wild-type p53, which is required

TABLE 1. Phenotypic characterization of progressed BALB/c 3T3 cells

Cell line ^a	Saturation density (10 ⁵ cells/dish) ^b		Development of actin cables ^c	Cloning efficiency in soft agar (%) ^d
	10% FCS	1% FCS		
BALB/c 3T3	26	4	++	0
BALB/c 3T3-cl4	46	15	++	0.01
BALB/c 3T3-cl10	90	20	+	5–10
SV3T3	100	40	±	60–80

^a For establishment of BALB/c 3T3-cl4 and BALB/c 3T3-cl10 cells, see Materials and Methods.

^b For a detailed description of growth conditions, see Materials and Methods. Cells were grown on 100-mm-diameter dishes.

^c ++, well-developed actin cable system; +, intermediately developed actin cables; ±, few and weakly developed actin cables.

^d A detailed description of the soft agar analysis is given in Materials and Methods. Values are means of two independent experiments. A colony consisting of more than 16 cells was considered positive.

for progression of these cells through the cell cycle (12, 73). The low levels of p53 in actively growing BALB/c 3T3 cells are the result of the very fast turnover of this protein, as determined by pulse-chase-labeling analyses (Fig. 1A). Graphic evaluation of Fig. 1A, shown in Fig. 1D, established that the p53 in these cells had a half-life of about 15 min.

Large T expression as such does not significantly influence the metabolic stability of p53 in BALB/c 3T3 cells (14). To analyze in detail the influence of complex formation with large T on the metabolic stability of p53 in these cells, BALB/c 3T3 cells were infected with SV40 at an MOI of 1 (for the definition of MOI in abortive infection, see Materials and Methods), and the metabolic stabilities of large T and of p53 complexed to large T in these cells were determined 24 and 48 h p.i. by pulse-chase experiments (see Materials and Methods). Figure 1B and C demonstrate that the p53 complexed to large T in these cells remained unstable. However, graphic evaluation (Fig. 1D) revealed a slight but significant overall increase in the half-life of p53 in infected versus uninfected 3T3 cells, from 15 min in uninfected cells to about 40 min in infected cells. This apparent increase in p53 stability is due mainly to a delayed decay of p53 during the first chase period of 15 min and may reflect a biphasic degradation of p53 complexed to large T, as suggested by others (62). Alternatively, continued labeling of p53 during the initial chase period due to an increased amino acid uptake as a consequence of viral infection, resulting in enlarged tRNA_{Met} and tRNA_{Cys} pools, might be envisioned. In this case, abortive SV40 infection would not have altered p53 stability at all. In any case, the apparent minor increase in metabolic stability of p53 after SV40 infection does not compare to the metabolic stability of p53 in SV40 stably transformed SV3T3 cells, derived from SV40 abortively infected BALB/c 3T3 cells via selection through agar cloning (see Materials and Methods): even after a 20-h chase period, about 70% of the pulse-labeled p53 in SV3T3 cells was still associated with large T (Fig. 2), indicative of a half-life of this p53 of over 20 h. These experiments thus confirm that the physical interaction of p53 with large T as such only minimally contributes to the extreme metabolic stability of p53 in SV40-transformed cells and suggest a correlation between expression of the transformed phenotype and metabolic stabilization of p53.

Metabolic stabilities of p53 in uninfected and SV40 abortively infected BALB/c 3T3 cells exhibiting a transformed phenotype after in vitro progression. According to the foregoing and previous experiments from our laboratory, metabolic

FIG. 1. Kinetic analysis of p53 degradation in uninfected and SV40 abortively infected BALB/c 3T3 cells. Uninfected BALB/c 3T3 cells (A) and BALB/c 3T3 cells abortively infected with an MOI of 1 at 24 (B) and 48 (C) h p.i. were pulse-labeled (p) and pulse-chase-labeled (c₁ to c₄) with 100 μ Ci of [³⁵S]methionine/cysteine (20-min pulse and 15-min [c₁], 30-min [c₂], 60-min [c₃], and 90-min [c₄] chases). Whole-cell lysates were immunoprecipitated for p53 with monoclonal antibody PAb122 (A) or for large T-p53 complexes with monoclonal antibody PAb108 (B and C). The immunoprecipitates were analyzed on an SDS–11.5% polyacrylamide gel subjected to fluorography. (D) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A to C.

stabilization of p53 complexed to large T seemed to correlate with a transformed phenotype of the large-T-expressing cells (14, 15). Whereas this condition by definition is met in SV40 stably transformed cells, SV40 abortively infected 3T3 cells do not display any of the characteristic features of the transformed phenotype, such as disorganization of actin cables, or any other gross morphological alterations (data not shown).

To test whether there is indeed a stringent correlation between metabolic stabilization of p53 complexed to large T and a transformed phenotype of the large-T-expressing cells, we wanted to analyze this process in cells displaying a transformed phenotype independent of any SV40-induced cellular alteration. Therefore, we established subclones of BALB/c 3T3 cells which had been progressed to a more transformed phenotype by cultivation of the cells at high cell densities (see Materials and Methods for details). Table 1 reveals that cells of these subclones showed a progressive increase in saturation densities when grown in medium containing either 10 or 1% serum, concomitant with a progressive disorganization of the actin cable system, and a significant increase in the ability to grow in soft agar. It is evident that cells in subclone 3T3-cl10, although not yet fully transformed like SV3T3 cells, displayed

FIG. 2. Metabolic stability of p53 in SV40-transformed BALB/c 3T3 cells (SV3T3 cl.4) developed from SV40 abortively infected cells. SV3T3 cl.4 cells were pulse-labeled (p) and pulse-chase-labeled (c₁ to c₄) with 100 μ Ci of [³⁵S]methionine/cysteine (1-h pulse and 4-h [c₁], 8-h [c₂], 16-h [c₃], and 24-h [c₄] chases). Whole-cell lysates were immunoprecipitated for large T-p53 complexes with monoclonal antibody PAb108. The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography (insert). Radiolabeled p53 in the fluorogram was evaluated by densitometer scanning.

an at least intermediate transformed phenotype, according to the nomenclature of Risser and Pollack (63). On the basis of previous experiments from our laboratory which had demonstrated that p53 in NIH 3T3 cells, considered to be already pretransformed (65), became metabolically stabilized upon abortive infection with SV40 (14), we expected a similar result in abortively infected progressed 3T3 cells.

As progression of 3T3 cells might have resulted in selection of cells expressing a stable mutant p53 (29), we first analyzed the metabolic stabilities of p53 in uninfected BALB/c 3T3, 3T3-cl4, and 3T3-cl10 cells. Figure 3 demonstrates that, in accordance with the phenotypes of these cells, the p53 in BALB/c 3T3 and 3T3-cl4 cells showed closely similar metabolic stability, with half-lives of 15 and 20 min, respectively. The apparent half-life (see also Fig. 1) of p53 in 3T3-cl10 cells had increased to approximately 40 min, i.e., to about the half-life of p53 in SV40 abortively infected BALB/c 3T3 cells (Fig. 1). This modest increase in metabolic stability, however, most likely cannot be attributed to a mutational alteration in the p53 gene, as the p53 in 3T3-cl10 cells by its reactivity with monoclonal antibody PAb246, specific for p53 in a wild-type conformation (84), and by its interaction with large T (see below) behaved like authentic wild-type p53 in 3T3 cells.

We next tested the influence of the cellular phenotype on the metabolic stability of p53 complexed to large T upon abortive infection of these cells. BALB/c 3T3, 3T3-cl4, and 3T3-cl10 cells were infected with SV40 at an MOI of 1 and analyzed for metabolic stabilization of p53 in complex with large T. Figure 4 reveals that, surprisingly, p53 in all cells analyzed was metabolically unstable and in all cells exhibited very similar half-lives of about 40 min. This result demonstrates the lack of any metabolic stabilization of p53 in BALB/c 3T3 cells during abortive infection with SV40, independent of the cellular phenotype. However, as p53 in BALB/c 3T3 cells stably transformed with SV40 (SV3T3 cells) becomes metabolically stabilized (Fig. 2), SV40 potentially can induce metabolic stabilization of p53 in 3T3 cells. We conclude that this induction does not simply correlate with a transformed phenotype of

FIG. 3. Analysis of metabolic stabilities of p53 in BALB/c 3T3 cells (A) and in vitro progressed 3T3-cl4 (B) and 3T3-cl10 (C) cells. Cells were pulse-labeled (p) and pulse-chase-labeled (c₁ to c₄) as described for Fig. 1. Whole-cell lysates were immunoprecipitated for p53 with monoclonal antibody PAb122. The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography. (D) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A to C.

the cells but rather requires the action of some unknown cellular mechanism which is induced or activated as a virus-specific event during SV40-induced cellular transformation.

Metabolic stabilization of p53 in pMEFd17 cells infected with SV40. The lack of any significant metabolic stabilization of p53 BALB/c 3T3 cells upon abortive infection with SV40 is not unique to these cells, as it was observed in a variety of established normal fibroblasts (14). However, in other cell types, lytic (19, 28, 78) or abortive (14, 17, 50) infection with SV40 readily induces metabolic stabilization of p53 complexed to large T, indicating that cells can differ in their requirements to mediate metabolic stabilization of p53 after SV40 infection.

Establishment of BALB/c 3T3 cells as normal fibroblasts from primary MEF had been achieved by a rigid cell culture protocol, selecting for strict contact inhibition of growth at a rather low saturation density (75). Therefore, we considered the possibility that establishment of 3T3 cells had somehow altered the cellular response to SV40 infection with regard to p53 stabilization. If this assumption is correct, pMEFd17 cells may constitute a better system for determining the factor(s) mediating metabolic stabilization of p53 during abortive infection with SV40.

pMEFd17 cells (see Materials and Methods) in passages 3 to 5 were infected with SV40 at an MOI of 1. The metabolic stabilities of p53 complexed to large T were determined 24 and 48 h p.i. and compared with that of p53 in uninfected pMEFd17 cells. Figure 5A shows that p53 in uninfected pMEFd17 cells was as unstable as p53 in BALB/c 3T3 cells, with a half-life of about 20 min. Upon abortive infection,

FIG. 4. Influence of the cellular phenotype on the metabolic stability of p53 complexed to large T after abortive infection with SV40. SV40-infected (MOI of 1) BALB/c 3T3 cells (A), 3T3-cl4 cells (B), and 3T3-cl10 cells (C) were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) as described for Fig. 1. Whole-cell lysates were immunoprecipitated for large T-p53 complexes with monoclonal antibody PAb108. The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography. (D) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A to C.

however, p53 became metabolically stabilized at 24 h p.i. (Fig. 5B and C), with no significant turnover during the 90-min chase period. We thus postulate that SV40 infection in pre-crisis MEF is able to readily induce or activate the cellular mechanism required to mediate metabolic stabilization of p53, whereas establishment of 3T3 cells from such primary cells had altered the cellular requirements mediating p53 stability upon SV40 infection.

Progressive induction of metabolic stabilization of p53 by SV40 during abortive infection of rat F111 fibroblasts. Our assumption that SV40 itself is able to induce a cellular mechanism which mediates metabolic stabilization of p53 complexed to large T could be further supported by analyzing metabolic stabilization of p53 in rat F111 fibroblasts abortively infected with SV40. Figure 6A shows that the p53 in uninfected F111 cells is as metabolically unstable as p53 in BALB/c 3T3 cells or in BALB/c pMEFd17 cells, with a half-life of about 20 min (Fig. 6D). However, 24 h after infection of these cells with SV40 at an MOI of 1, the half-life of the p53 complexed to large T had increased to about 70 min (Fig. 6B and D), already

FIG. 5. Metabolic stabilization of p53 in pMEFd17 cells infected with SV40. Uninfected cells (A) and SV40 abortively infected cells (MOI of 1) at 24 (B) and 48 (C) h p.i. were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) as described for Fig. 1. Whole-cell lysates were immunoprecipitated for p53 with monoclonal antibody PAb22 (A) or for large T-p53 complexes with monoclonal antibody PAb108 (B and C). The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography. (D) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A to C.

marking a significant metabolic stabilization of p53. At 48 h p.i., the p53 complexed to large T had become significantly more stabilized, with over 90% of the p53 radioactively labeled during the pulse still being associated with large T after the 90-min chase period (Fig. 6C and D). Thus, the metabolic stability of complexed p53 in SV40 abortively infected F111 cells gradually was approaching the stability of p53 in F111 stably transformed cells [e.g., FR(wt648) cells].

Free p53 in SV40 abortively infected cells remains metabolically unstable. p53 in SV40 transformed cells consists of two populations, p53 bound to large T and free p53 (i.e., p53 not bound to large T), which exhibit similar metabolic stabilities (13, 14). As the metabolic stabilities of p53 complexed to large T differed between abortively infected 3T3 and pMEFd17 cells, we analyzed the free p53 in these cells, which constitutes about 10 to 20% of the total p53 in these cells (data not shown). 3T3 and pMEFd17 cells were infected with SV40 at an MOI of 1, pulse-chase-labeled with [^{35} S]methionine/cysteine as described above, and analyzed for free p53 by sequential immunoprecipitation as described previously (13, 14). Extracts were first cleared from large T-p53 complexes by immunoprecipitation with the large-T-specific monoclonal antibody PAb419, followed by immunoprecipitation for p53 with the p53-specific

FIG. 6. Metabolic stabilization of p53 in rat F111 fibroblasts during abortive infection with SV40. Uninfected cells (A) and SV40 abortively infected cells (MOI of ~ 1) at 24 (B) and 48 (C) h p.i. were pulse-labeled (p) and pulse-chase-labeled (c_1 to c_4) as described for Fig. 1. Whole-cell lysates were immunoprecipitated for p53 with monoclonal antibody PAb421 (A) or for large T-p53 complexes with monoclonal antibody PAb419 (B and C). The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography. The coprecipitating proteins migrating above (A to C) or below (A) p53 are unrelated to p53, as demonstrated by peptide analysis (data not shown). (D) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A to C.

monoclonal antibody PAb421. Figure 7A and B demonstrate that free p53 in 3T3 cells as well as free p53 in pMEFd17 cells was metabolically unstable and exhibited the same metabolic stabilities as the p53 in the parental, uninfected cells (Fig. 7C; see also Fig. 1A and C and Fig. 5A and C), with a half-life of about 15 to 20 min. Similarly, we found that free p53 in abortively infected F111 cells also remained metabolically unstable (data not shown). Thus, the metabolic stability of free p53 in SV40 abortively infected cells appears to be completely unaffected by alterations in the metabolic stability of p53 complexed to large T in these cells. This finding (i) supports our previous conclusion that complexed and free p53 constitute two different p53 populations in these cells (13, 14) and (ii) suggests that metabolic stabilization of both populations of p53 is a two-step process during SV40 transformation.

Metabolic stabilization of p53 during abortive infection with SV40 correlates with an increased transformation efficiency. The cellular systems characterized above were used to analyze the functional relevance of p53 stabilization for SV40-induced transformation. Metabolic stabilization in abortively infected

FIG. 7. Analysis of free p53 in SV40 abortively infected cells remains metabolically unstable. SV40 abortively infected BALB/c 3T3 cells (A) and pMEFd17 cells (B) at 48 h p.i. were pulse-labeled (p) or pulse-chase-labeled (c_1 to c_4) as described for Fig. 1. Whole-cell extracts were analyzed for free p53 by sequential immunoprecipitation as described in the text. Extracts were first cleared from large T and large T-p53 complexes by immunoprecipitation with the large-T-specific monoclonal antibody PAb419, followed by immunoprecipitation for free p53 with the p53-specific monoclonal antibody PAb421. The immunoprecipitates were analyzed on an SDS-11.5% polyacrylamide gel subjected to fluorography. (C) Graphic evaluation of densitometer scans of radiolabeled p53 in fluorograms shown in panels A and B.

pMEFd17 cells grossly enhances the steady-state level of p53 in these cells compared with p53 levels in abortively infected BALB/c 3T3 cells. This could be demonstrated by Western blot analysis. Lanes a and b in Fig. 8A demonstrate an approximately 10- to 20-fold difference in p53 levels between 3T3 and pMEFd17 cells, with both types of cells expressing rather similar levels of SV40 large T (Fig. 8B). If, as we postulate, enhanced levels of p53 are advantageous for SV40-mediated transformation, pMEFd17 cells, despite being primary cells, might be more easily transformable by SV40 than 3T3 cells.

BALB/c 3T3 and pMEFd17 cells were infected with SV40 at an MOI of 1. After 1 day, cells were transferred into soft agar as described in Materials and Methods and scored for growth of transformed colonies after about 2 weeks. Table 2 shows that infection of 3T3 cells with SV40 at an MOI of 1 resulted in a transformation efficiency of approximately 0.01%, whereas the transformation efficiency of SV40-infected pMEFd17 was increased by factor of 10. To exclude the possibility that these differences in transformation efficiencies reflected the analysis of precursors versus established cells rather than differences in the cells abilities to stabilize p53, we analyzed established normal rat fibroblasts differing in the ability to stabilize p53 upon SV40 infection. As documented in Table 2, all uninfected

FIG. 8. Steady-state levels of SV40 large T (A) and p53 (B) in SV40 abortively infected pMEFd17 cells and BALB/c 3T3 cells at 48 h p.i. Whole-cell extracts of pMEFd17 cells (lane a) and BALB/c 3T3 cells (lane b) were immunoprecipitated for total p53 with monoclonal antibody PAb421 (B) or for large T-p53 complexes with monoclonal antibody PAb419 (A). Immunoprecipitates were analyzed by Western blotting as described in Materials and Methods. Panels A and B were exposed for different periods of time.

cells were unable to grow in soft agar. These cells also showed no other readily detectable marker of phenotypic transformation (data not shown). SV40 had a very low transformation efficiency in FR3T3 cells, correlating with the fact that abortive infection of these cells did not significantly increase the metabolic stability of their p53. In contrast, abortive infection of F111 cells with SV40 resulted in a rather high efficiency of transformation, correlating with an increased stability of p53 after abortive infection (Fig. 6). The differences in transformation efficiencies of SV40 in cells stabilizing or not stabilizing p53 complexed to large T upon abortive infection were also observed when formation of dense foci was used as a transformation marker (data not shown), suggesting that the observed differences in transformation efficiencies were not due to the selection parameter applied.

DISCUSSION

In this study, we demonstrate that metabolic stabilization of p53 complexed to large T during SV40-mediated cellular transformation is a cellular event, specifically induced by SV40.

This process does not depend simply on the cells assuming a transformed phenotype, as complexed p53 was stable in abortively infected precrisis MEF (which by definition are not transformed) but remained unstable in abortively infected BALB/c 3T3-derived cells (3T3-cl10) exhibiting at least some characteristics of transformed cells. As metabolic stabilization of p53 in these cells, as in the original 3T3 cells, was observed only after stable transformation with SV40, we conclude that the cellular processes leading to metabolic stability of p53 during SV40 transformation are specifically induced by SV40 and not merely a corollary of SV40 transformation. In SV40 abortively infected, as in SV40-transformed cells, p53 in complex with large T comprises the majority of total p53 (approximately 80% of total p53 in SV40 abortively infected cells; data not shown). As reported previously (14), we also found in this study that the residual free p53 in such cells remained metabolically unstable, regardless of whether p53 in complex with large T became stabilized during abortive infection. Since free p53 in SV40 stably transformed cells is metabolically stable (13), metabolic stabilization of p53 complexed to large T in SV40 abortively infected cells marks an initial alteration of cellular pathways controlling p53 stability during SV40 transformation. We previously demonstrated that metabolic stabilization both of p53 complexed to large T and of free p53 correlated with the establishment of stable transformation and with maintenance of the transformed phenotype (14, 15). Thus, our finding that free p53 in SV40 abortively infected cells did not become stabilized might reflect that SV40 abortively infected cells did not display any obvious signs of morphological transformation, such as disorganization of actin cables or loss of density-inhibited growth (data not shown). This suggests that metabolic stabilization of free p53 may constitute an indicator for the establishment or maintenance of stable transformation by SV40. Like stabilization of complexed p53, stabilization of free p53 correlated with the expression of a functional large T (14, 15). Therefore, we assume that metabolic stabilization of free p53 during establishment of cellular transformation is SV40 controlled.

Analysis of the metabolic stabilities of free p53 in SV40 abortively infected 3T3 and pMEFd17 cells also provided evidence for a slight but significant stabilization of p53 complexed to large T in abortively infected 3T3 cells. The half-life of the free p53 in such cells remained at about 15 min, whereas that of complexed p53 was about 40 min. Therefore, one has to

TABLE 2. Transformation efficiencies of SV40 in BALB/c mouse 3T3, in pMEFd17, and in rat FR3T3 and F111 cells

Cells	Half-life of p53	Avg no. of colonies of transformed cells in soft agar ^a at input of:			Efficiency of colony growth (%)
		10 ⁵	10 ⁴	10 ³	
BALB/c 3T3					
Uninfected (control)	15 min	2	0	0	0.002
SV40 infected (MOI ~ 1)	40 min	14	1–2	0	~0.01
Primary BALB/c MEF					
Uninfected (control)	20 min	0	0	0	0
SV40 infected (MOI ~ 1)	>90 min	100	8	1	~0.1
SV3T3	>24 h	— ^b	—	600–800	60–80
FR3T3					
Uninfected (control)	20 min	0	0	0	0
SV40 infected (MOI ~ 1)	40 min	6	0	0	0.006
F111					
Uninfected (control)	20 min	0	0	0	0
SV40 infected (MOI ~ 1)	>90 min	—	105	11	~1

^a See Materials and Methods.

^b —, not evaluable.

attribute this increase to a slower degradation of the complexed p53. This excludes the alternate possibility of a continuation of p53 labeling during the initial phase of the chase as a result of an enlarged tRNA_{Met} and tRNA_{Cys} pool in these cells due to an increased amino acid uptake in infected cells. Evidence for a biphasic degradation of p53 has been provided (62). Similarly, we conclude that the increase in half-life of p53 in progressed 3T3-cl10 cells to about 40 min also reflects a partial stabilization of p53 in these cells as a result of phenotypic alterations. This conclusion is supported by Western blot analyses of p53 steady-state levels, which showed an increase in p53 levels in progressed 3T3-cl10 and abortively infected 3T3 cells (data not shown).

Nothing is known so far about the possible molecular mechanism(s) leading to SV40-mediated metabolic stabilization of either complexed or free p53 during SV40 transformation. For complexed p53, such a mechanism could act either on large T or on p53. Thus, an SV40-induced cellular mechanism could modify either large T or p53 by posttranslational mechanisms (e.g., phosphorylation) which alter the turnover of p53 in complex with large T. In this regard, it has been suggested that SV40 induces a specific cellular kinase leading to altered phosphorylation of p53 in complex with large T (53, 69), but the evidence provided for this potentially interesting mechanism so far is circumstantial (56). An alternate possibility which has to be considered especially for metabolic stabilization of free p53 is that SV40 directly interferes with p53 metabolism, either by inducing a cellular factor actively mediating p53 stability or by down-regulating the p53 degradation pathway. The prominent role for cellular processes in stabilizing p53 has been demonstrated previously by experiments analyzing metabolic stabilization of wild-type p53 and of various p53 mutants introduced into mouse fibrosarcoma cells (26). The involvement of so far unidentified cellular factors in p53 stabilization can also be deduced from the recent finding that the adenovirus E1A 12S protein induces metabolic stabilization of p53 (51), an event previously ascribed to complex formation of p53 with the adenovirus E1B 55-kDa tumor antigen (68). The E1A protein exhibits well-documented transactivating and transrepressing properties (reviewed in reference 71). Therefore, it is likely that the E1A protein mediates p53 stability by either inducing or repressing a cellular function involved in p53 metabolism. We assume that SV40 induces p53 stabilization by a similar mechanism. This assumption is strongly supported by our finding that p53 stabilization in abortively infected F111 cells was a progressive event during the course of infection. The gradual increase of p53 stability in these cells was unrelated to large T levels in these cells and thus could be well explained by the gradual accumulation or repression of a cellular factor altering p53 turnover. Induction or repression of such a cellular factor seems to be directly induced by SV40, as various transformation-defective large T proteins (e.g., tsA mutant large T) at the nonpermissive temperature do not induce or maintain metabolic stabilization of p53 (14, 15, 50).

Our hypothesis that metabolic stabilization of p53 is an SV40 function required for cellular transformation is further supported by our comparative analysis of the transformation efficiency of SV40 in various cells differing in the ability to mediate p53 stabilization during abortive infection. We found a close correlation between metabolic stabilization of p53, resulting in higher levels of p53, and transformation efficiency, independent of cell type. This finding is in line with our previous suggestion that wild-type p53 in SV40 transformation acts as a cooperating oncogene, perhaps by assuming properties of a gain-of-function mutant p53 (10). To date, neither the

postulated oncogenic function of stabilized p53 in SV40 transformation nor the SV40 function mediating metabolic stabilization of p53 has been identified. However, the cellular systems described above, in conjunction with the large panel of SV40 early mutants, should allow us to answer these questions.

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