Complex Formation between the Lymphotropic Papovavirus Large Tumor Antigen and the Tumor Suppressor Protein p53

HOLLY SYMONDS, JIANDONG CHEN, AND TERRY VAN DYKE*

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received 30 May 1991/Accepted 18 July 1991

The simian B-lymphotropic papovavirus (LPV) encodes a large tumor antigen (T antigen) which is 45% identical to both the simian virus 40 (SV40) and the polyomavirus (PyV) large T antigens. In transgenic mice, the transforming properties of the LPV T antigen are similar to those of the SV40 T antigen. However, little is known about its biochemical activities. Since SV40 T antigen forms a complex with and stabilizes the host cell tumor suppressor protein p53 while the PyV large T antigen does not, we characterized the LPV T antigen for its ability to complex p53. We demonstrate an association between LPV T antigen and p53 in both a tumor-derived cell line and BALB/c 3T3 cells transformed in culture. A third protein of approximately 68 kDa which was found associated with the LPV T antigen-p53 complex in tumor-derived cells appears to be heat shock protein 70 (hsp70). The half-life of p53 in all LPV T-antigen-transformed cells was extended significantly; i.e., it was 3 to 7 h compared with 19 minutes in BALB/c 3T3 cells. The half-life of the LPV T antigen itself was 5 to 9 h depending on the cell line origin. That p53 was stabilized because of association with LPV T antigen and not because of mutation was demonstrated with the p53 conformation-dependent monoclonal antibody PAb246. This antibody distinguishes between wild-type p53 (PAb246⁺) and mutant, oncogenic p53 (PAb246⁻). Sequential immunoprecipitation showed all detectable p53 to be of the PAb246⁺ class in each LPV-transformed cell line, suggesting that the stable p53 was indeed wild type.

DNA tumor virus transforming proteins form stable complexes with several cellular proteins, some of which appear to act normally to regulate cell growth (for reviews, see references 22, 26-28, and 30). Characterization of the cellular targets and their normal functions should help elucidate the mechanisms used by these viral proteins to disrupt cell growth control. Among the papovaviruses, the simian virus 40 (SV40) large tumor antigen (T antigen) has been extensively studied at both the biochemical and genetic levels with respect to cell transformation (for reviews, see references 34 and 48). The papovavirus large T antigens can be divided into two different classes with respect to both transforming properties and structural characteristics. The SV40 protein, representing one class, is sufficient to transform many cells in culture (34) and efficiently induces tumors in a variety of cell types in transgenic mice (reviewed in reference 18). The SV40 T antigen forms a complex with two tumor suppressor proteins, pRb (9) and p53 (23, 29; reviewed in references 27 and 30). Genetic evidence suggests that each of these T antigen binding activities has a role in the transformation of cultured cells, although each activity is also dispensable for the transformation of certain cell types, depending on the assay used (8, 33, 46, 47). While the polyomavirus (PyV) large T antigen immortalizes cells in culture, it is not sufficient to transform them but rather requires additional activities such as those provided by the PyV middle T antigen (38). PyV large T antigen binds to the pRb protein (11), but it fails to bind to p53 (50). Mutants of PyV T antigen which do not bind to pRb fail to immortalize primary embryo fibroblasts (25).

Relatively little is known about the transforming functions of a related protein, the lymphotropic papovavirus (LPV) T antigen. LPV was originally isolated from African green monkey B lymphocytes (53) and grows only in monkey and

human B-lymphoblastoid lines in culture (3). The LPV nucleotide sequence (15, 36) indicates that the large T antigen shares 45% sequence identity with both SV40 and PyV T antigens (10). LPV T antigen is structurally similar to PyV T antigen in that it contains additional amino acids within the amino terminal domain not present in SV40 T antigen and lacks the carboxyl-terminal host range domain present in SV40 T antigen. The LPV early region does not encode a middle T antigen (36). In transgenic mice, the SV40 and LPV T antigens appear to have similar transforming properties, since they both induce choroid plexus tumors when regulated by their own promoters (5, 6). Furthermore, using hybrid transgenes which carry one viral T-antigen-coding region driven by the promoter of the other, we have recently shown that the two T antigens cause indistinguishable proliferative disorders in many tissues in transgenic mice (7). These results suggest that the two proteins may have common cellular targets. The LPV T antigen has been shown to bind pRb in vitro (11), but other host protein binding properties, such as complex formation with p53, have not been reported.

p53 was initially discovered as a cellular protein bound to SV40 T antigen in transformed cells (23, 29). p53 is present in low levels in nontransformed cells (for a review, see reference 22) and has a half-life of about 6 to 20 min, depending on the cell type (26-28, 30). However, a 10- to 100-fold elevation of p53 steady-state levels occurs in certain tumors and in cells transformed by a variety of means, including viral infection, chemical treatment, and transfection of oncogenes (22, 26-28, 30). The complex formed between SV40 T antigen and p53 also correlates with an increased level of p53 evident in immunoprecipitations and immunoanalyses (26-28, 30, 35), and pulse-chase studies indicate that the increase is a result of protein stabilization (for reviews, see references 22 and 30).

The similar transforming abilities of LPV and SV40 T antigens in vivo would predict that, if the p53 binding

* Corresponding author.

property were important for this process, LPV T antigen would also bind to p53. We tested this prediction by analyzing the interaction between LPV T antigen and p53 in tumor cells and in cells transformed in culture.

MATERIALS AND METHODS

Cell lines. LCP726 cells were derived from a choroid plexus tumor from a transgenic mouse which harbored the LPV early region (6). Tumor tissue was removed, dissociated with trypsin, and expanded in Dulbecco's high glucose modified essential medium (DMEM; Hazelton) with 10% fetal calf serum. SVT2, an established SV40-transformed cell line (1), was maintained in the same medium. SLT/A4 and SLT/B5 cells were expanded from foci after the CaPO_4 transfection (45) of BALB/c 3T3 cells with pSLT, a plasmid which directs production of the LPV T antigen under the control of the SV40 early promoter (7). The SLT/B56 cell line was derived from a soft-agar clone of SLT/B5. SVE1/C2 cells were BALB/c 3T3 cells transformed by CaPO_4 transfection with pSVE1, a plasmid which directs production of the wild-type SV40 T antigens via their own promoter. C3H10T $_{1/2}$ -derived M53-1 cells (a kind gift from A. Srinivasan and J. Pipas, University of Pittsburgh, Pittsburgh, Pa.) express a mutant p53 protein with a valine at amino acid position 135 (27, 28). BALB/c 3T3 cells and derivatives were maintained in high-glucose DMEM (Hazelton) supplemented with 10% calf serum and were maintained at 37°C in 5% CO_2 .

Antibodies. Polyclonal anti-SV40 T antigen ascites was obtained from Syrian hamsters as described by Lanford and Butel (24). Polyclonal anti-LPV T-antigen hamster serum was a generous gift from W. Deppert (Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie an der Universität Hamburg, Hamburg, Germany). Polyclonal anti-heat shock protein 70 (hsp70) serum was obtained from D. DeFranco (University of Pittsburgh). Anti-p53 monoclonal antibodies PAb421 and PAb246 have been described previously (19, 52). Monoclonal antibodies directed against various regions of the SV40 T antigen were tested for the recognition of LPV T antigen but failed to immunoprecipitate this protein. The following antibodies have been described previously: PAb101, PAb102, PAb106, PAb107, and PAb113 (17) and PAb405, PAb416, and PAb419 (19).

Radiolabeling and immunoprecipitation. Cells were seeded in 10-cm-diameter dishes and incubated in subconfluent monolayers overnight. The cells were washed in phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , and 1.75 mM KH_2PO_4 , pH 7.3) and methionine-free DMEM supplemented with 2% fetal calf serum that had been dialyzed for 24 h in PBS. The cells were then labeled with 100 μCi of ^{35}S -trans label (ICN Biomedicals, Inc.). Trans label contains approximately 70% L-[^{35}S]methionine, 15% L-[^{35}S]cysteine, 7% L-[^{35}S]methionine sulfone, 3% L-[^{35}S]cysteic acid, and 5% trace ^{35}S compounds. The standard labeling time, with the exception of the pulse-chase experiments, was 1.5 h. Cells were then rinsed with PBS, scraped from the plates, and centrifuged at $850 \times g$ for 4 min. Cell pellets were resuspended in a lysis buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40. A cocktail of protease inhibitors (Boehringer Mannheim Biochemicals), including 25 mg of phenylmethylsulfonyl fluoride per ml, 5 mg of aprotinin per ml, 0.3 mg of EDTA- Na_2 per ml, 0.5 mg of leupeptin per ml, and 0.7 mg of pepstatin per ml, was added to the lysis buffer. Cells were lysed by vortexing, and supernatants were obtained after centrifugation at $700 \times g$.

Protein concentrations were determined by the method of Bradford (4) with the concentrated dye reagent from Bio-Rad Laboratories and were then adjusted to be approximately equivalent by dilution in lysis buffer. The amounts of labeled protein in the extracts were determined after precipitation in 5% trichloroacetic acid at 4°C. Samples of each extract (300 μl) were preabsorbed with heat-fixed *Staphylococcus aureus* A cells (Boehringer Mannheim). Primary antibody was added to the cleared extract and incubated at 4°C for 1 h. Twenty microliters of *S. aureus* cells was added, and the incubation was continued for 20 min. The immunocomplex was then pelleted and washed twice with SNTE (5% sucrose, 0.1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris-HCl [pH 7.4], and 5 mM EDTA) and once with NTE (50 mM NaCl, 10 mM Tris-HCl [pH 7.4], and 1 mM EDTA). The pellets were resuspended in protein sample buffer (5% glycerol, 1% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue, 5% β -mercaptoethanol, and 0.0625 M Tris-HCl [pH 6.8]), boiled for 5 min, and applied to a 10% polyacrylamide denaturing gel (46). The destained gels used for data in Fig. 1 to 4 were submerged in En 3 Hance (DuPont-NEN Research Products) for 30 min. The gels shown in Fig. 5 were directly exposed to Kodak film without fluorography.

Sequential immunoprecipitation. BALB/c 3T3, M53-1, LCP726, and SLT/B5 extracts were first immunoprecipitated with PAb246 or with PAb421 as described above, except that twice as much of each component was used in the initial reactions. The supernatants were cleared once more with the original antibody, and the resulting supernatants were divided into two equal aliquots and then incubated with either PAb421 or PAb246. The immunocomplexes were washed and analyzed as described above.

Pulse-chase radiolabeling. Subconfluent monolayers of cells were labeled with 250 μCi of ^{35}S -trans label (ICN) per 10-cm-diameter plate for 15 min as described above. After four washes with PBS, cold methionine-containing DMEM supplemented with 10% calf serum was added to the plates, and the transformed cells were collected immediately (pulse) or further cultured for 0.5, 1, 6, 12, 18, or 24 h (chase). Shorter chase periods of 15, 30, 45, 60, and 120 min were used for the BALB/c 3T3 cells. The cells were washed with PBS, scraped off the plates, lysed, and immunoprecipitated with PAb421 or hamster anti-T serum as described above. To quantitate the amounts of specific labeled proteins in the pulse-chase experiment and in the sequential immunoprecipitation reactions, the protein bands in dried acrylamide gels were analyzed with the AMBIS densitometric program (Automated Microbiology Systems Inc.). The half-life was determined from a least-squares fit of the data, which is shown in Fig. 3 and 4.

RESULTS

p53 is complexed with LPV T-antigen in transformed cell lines. To determine whether LPV T antigen can form a complex with p53, we performed a variety of coprecipitation experiments with polyclonal antibodies to SV40 T antigen and to LPV T antigen and a monoclonal antibody to p53, PAb421. We first analyzed LCP726 cells, an established culture derived from an LPV-induced choroid plexus tumor (6). The 84-kDa LPV T antigen was immunoprecipitated either with the polyclonal serum to SV40 tumor antigens (Fig. 1A, lane 14) or with PAb421 (lane 15) and thus appeared to be present in a complex with p53. As was the case for SV40 T antigen (lanes 2 and 5 to 7), the LPV-T-antigen-transformed cells contained an increased level of p53

FIG. 1. Complex formation between LPV T antigen and p53 in tumor-derived cells. (A) The LPV-induced tumor-derived cell line LCP726 (lanes 13 to 16), nontransformed BALB/c 3T3 cells (lanes 9 to 12), and the SV40-transformed cell lines SVE1/C2 (lanes 4 to 8) and SVT2 (lanes 1 to 3) were incubated with 35 S-labeled amino acids for 1.5 h. The proteins were immunoprecipitated from extracts with either polyclonal hamster anti-SV40 T-antigen serum (α SV40-T, lanes 5, 10, and 14), one of two different anti-p53 monoclonal antibodies (PAb246, lane 7, and PAb421, lanes 2, 6, 11, and 15), or hamster polyclonal anti-hsp70 serum (α hsp70, lanes 3, 8, 12, and 16). This anti-hsp70 serum also precipitated several nonspecific cellular proteins which were evident in all of the extracts, including the control BALB/c 3T3 cell line, and which therefore were not related to the LPV T antigen (lanes 3, 8, 12, and 16). Proteins were resolved on an SDS-10% polyacrylamide gel and detected by autoradiography. (B) Total LPV T antigen present in LCP726 cells was immunoprecipitated with hamster anti-LPV T-antigen serum (α LPV-T, lane 2). As a control (c), the BALB/c 3T3 cell extract was immunoprecipitated with hamster α LPV-T (lane 1). Reactions using normal mouse serum (NMS, lane 3), α SV40-T serum (lane 4), and PAb421 (lane 5) are also shown. (C) Immunoprecipitation of LPV T antigen from a single cell clone of LCP726 (lanes 2 to 4). All samples were analyzed on a single gel. Molecular sizes of marker proteins (M) are given in kilodaltons at the left of panel A.

protein (lanes 14 and 15) compared with that of the BALB/c 3T3 nontransformed line (lanes 10 and 11). Immunoprecipitation with a polyclonal serum directed against LPV T antigen showed a similar result (Fig. 1B, lane 2) and revealed the total amount of LPV T antigen in the extracts, a portion of which was not detected with the anti-SV40 serum (compare lanes 2 and 4). The anti-LPV T-antigen serum recognized very little p53 directly (Fig. 2; compare lanes 2 and 3). In subsequent experiments, this serum was used to analyze LPV T antigen. The complex between p53 and LPV T antigen was also evident in LCP726TJ, a single-cell clone of LCP726 (Fig. 1C, lanes 2, 3, and 4).

In addition to LPV T antigen and p53, a third prominent protein of 68 kDa was immunoprecipitated from the LCP726 extract with either PAb421 or anti-T-antigen sera (Fig. 1A, lanes 14 and 15 and Fig. 1B, lanes 2, 4, and 5). This protein

was not pronounced in similar experiments using the SV40-transformed lines SVT2 and SVE1/C2 (Fig. 1A, lanes 2, 5, and 6). Previous studies have shown that hsp70 and its constitutively expressed family member hsc70 coimmunoprecipitate with certain mutant forms of p53 (13, 20, 37). Recent studies have also reported that SV40 T antigen can bind to both hsp70 and hsc70 (31, 42). We therefore analyzed the LPV-transformed cell extracts for the presence of hsp70 in the T antigen-p53 complexes with a polyclonal hamster serum specific for hsp70. This antibody also immunoprecipitated LPV T antigen, p53, and a protein which migrates with the 68-kDa species observed in the previous reactions with anti-T-antigen or anti-p53 antibodies (Fig. 1A, lane 16). The hsp70 antibody did not precipitate either SV40 T antigen or p53 from the SVT2 or SVE1/C2 extracts, nor did it detect the BALB/c 3T3 p53 (Fig. 1A, lanes 3, 8, and 12).

The LCP726 line was generated from tumor tissue and thus represents a unique isolate with no available parental control. While use of this cell line demonstrates the ability of LPV T antigen to complex with p53, we were interested in determining whether the complex was a general property of LPV-transformed cells and in measuring the apparent stabilization of p53. We created additional LPV-transformed cell populations by transfecting BALB/c 3T3 cells with a recombinant gene (SLT) that carries the LPV-T-antigen-coding sequences regulated by the SV40 control region. Independent foci were expanded, and the resulting cultures (SLT/A4 and SLT/B5) were analyzed for complex formation between LPV T antigen and p53, as had been carried out for the LCP726 cells. A soft-agar clone of SLT/B5, SLT/B56, was also analyzed. As a control, the SV40-transformed line SVE1/C2 was derived in the same experiment. In all cases, LPV T antigen was detected in a complex with p53, and increased levels of p53 were detected compared with levels in parental BALB/c 3T3 cells (Fig. 2). The amount of p53 in the SLT/B5 and SLT/B56 cell extracts is approximately 10-fold higher than that present in the nontransformed BALB/c 3T3 cells and is similar to the level found in the SV40-transformed SVE1/C2 cells (Fig. 2, lanes 11 to 13 and 17 to 19). The association of hsp70 with LPV T antigen and p53 was not detected in these BALB/c 3T3 transformed lines (Fig. 2, lanes 14 and 20).

In all LPV-induced BALB/c 3T3 transformants, the amount of labeled LPV T antigen is lower than the amount present in LCP726 cells and is also submolar to the amount of p53 in the complex (Fig. 2). Repeated immunoprecipitations with varied experimental conditions, including protein labeling times and salt and protease inhibitor concentrations, gave identical results (data not shown). Although the pSLT-transformed cell extracts contained a similar amount of p53, sequential immunoprecipitations using both anti-T-antigen sera in antibody excess verified that the pSLT-transformed cell extracts contained much less T antigen than was found in LCP726 cells or in the corresponding SV40-transformed pSVE1/C2 cells.

p53 is stabilized in LPV T-antigen-transformed cells. We analyzed the stabilities of both SV40 and LPV T antigens, of p53 in the transformed cells described above, and of p53 in the parental BALB/c line. Transformed cells were pulse-labeled for 15 min and either lysed immediately or chased with cold amino acids for 1, 6, 12, 18, or 24 h. BALB/c 3T3 cells were also pulse-labeled for 15 min, but shorter chase periods of 15, 30, 45, 60, and 120 min were used. The extracts were immunoprecipitated with PAb421 or with the serum specific for either SV40 T antigen or LPV T antigen, depending on the cell line under study. We reproducibly

FIG. 2. LPV T antigen complexes with p53 in cells transformed in culture. SLT/B5 (lanes 10 to 15) and SLT/B56 (lanes 16 to 21) were independently derived from foci induced after the transfection of BALB/c 3T3 cells with the SLT gene (the LPV T-antigen-coding region under SV40 regulation). Other cell lines are as indicated. T antigens and p53 were immunoprecipitated as described in the legend to Fig. 1. All samples were analyzed on a single gel. Extracts for lanes 9, 15, and 21 were lysed under different conditions by using radioimmunoprecipitation assay lysis buffer containing 1% deoxycholate. The molecular mass markers (in kilodaltons) and the positions of SV40 T antigen (SV40-T), LPV T antigen (LPV-T), p53, and the 68-kDa protein are indicated.

observed that over 50% of the labeled SV40 T antigen from SVE1/C2 cells was still present after 24 h (Fig. 3). In a parallel pulse-chase experiment, the half-life of LPV T antigen was about 9 h in the LCP726 line and 5.5 h in the SLT/B5 clone and its soft-agar derivative, SLT/B56 (Fig. 3).

Having obtained BALB/c 3T3 cells that were transformed by LPV T antigen, we were able to assess the stability of p53 in these cells compared with its stability in the parental control. The half-life of p53 in the BALB/c 3T3 cell line (Fig. 4a) was approximately 19 min, which is consistent with the previously reported value of 20 min for this line (39). The p53 half-life in control cells transformed by SV40 T antigen (SVE1/C2 cells) was 8.5 h, approximately a 27-fold increase (Fig. 4b). These results suggest that SV40 T antigen leads to p53 stabilization as previously documented (for a review, see references 26–28; 40). In parallel pulse-chase experiments, the half-life of p53 was also assessed in the LPV-transformed lines LCP726, SLT/A4, SLT/B5, and SLT/B56. In LCP726 cells (tumor derived), the p53 half-life was approximately 7.0

h (Fig. 4c), a 22-fold increase over the half-life of p53 in the BALB/c 3T3 line. The pSLT-transformed cells also contained a stable population of p53; 50% of the p53 protein remained after 3.0 to 4.5 h, depending on the isolate (Fig. 4d). These data provide evidence that, as with SV40 T antigen, complex formation between LPV T antigen and p53 leads to p53 stabilization.

Stabilized p53 in LPV-transformed cells is wild type. The p53 protein appeared to be stabilized as a result of complex formation with LPV T antigen in the transformed cell lines. However, an alternative explanation could be that p53 was stabilized in each case by mutation (27, 28). A variety of monoclonal antibodies which recognize p53 have been characterized (16, 19, 49, 52). In particular, PAb246 has been shown to recognize wild-type but not mutant forms of p53 (for a review, see reference 22; 52). Experiments using PAb246 suggest that p53 undergoes a common conformational alteration associated with the loss of the PAb246 epitope when mutated to produce a dominant oncogenic activity regardless of the type of mutation involved (22, 52). PAb421, which was used in all previous experiments, recognizes both wild-type and stabilized mutant p53. We therefore utilized the specific binding activity of PAb246 to determine whether the p53 present in LPV-transformed cells was of the wild-type conformation (and thus stabilized as a result of LPV T-antigen interaction and not fortuitously by mutation). Sequential immunoprecipitation with PAb246 and PAb421 was carried out for the LCP726 and SLT/B5 cell lines. As a control, M53-1, a cell line which expresses mutant p53 (valine 135) was used to demonstrate the presence of two populations of p53 under the same conditions. Immunoprecipitation of the M53-1 extract with PAb246 detected only a subpopulation of p53 (approximately 25% of the total) which was not recognized by further incubation with PAb246 (lane 14) but which reacted with PAb421 (lane 15). Such a PAb246⁺, PAb421⁺ population of p53 was not present in either the LCP726 or SLT/B5 cells (Fig. 5B); rather, all detectable p53 was recognized by the PAb246 antibody.

DISCUSSION

Although LPV was first isolated more than 10 years ago (53), little is known about the transforming properties of its

FIG. 3. Metabolic stability of LPV T antigen. SVE1/C2 (□), LCP726 (○), and SLT/B56 (●) cells were pulse-labeled, and the label was chased as described in Materials and Methods. Equal amounts of total protein were immunoprecipitated with polyclonal anti-SV40 T-antigen or anti-LPV T-antigen antibody. The amount of each T antigen in the gel was quantified by using the AMBIS radioanalysis system as described in Materials and Methods. Half-lives were determined from a least-squares fit of the data.

FIG. 4. Metabolic stability of p53 in BALB/c 3T3 cells (a), SVE1/C2 cells (b), LCP726 cells (c), and SLT/A4 and SLT/B56 cells (d). For each of the different time points, transformed cells were labeled with ^{35}S -labeled amino acids for 15 min (pulse) and chased for 1, 6, 12, 18, or 24 h. The BALB/c 3T3 cells were labeled for shorter chase periods of 15, 30, 45, 60, and 120 min. Equal amounts of total protein were incubated with the monoclonal antibody PAb421. Half-lives were analyzed as described in the legend to Fig. 3.

large T antigen. Studies with transgenic mice which have shown that both LPV and SV40 T antigens induce similar proliferative disorders (5–7) suggest that the two viral antigens may have biochemical activities in common which lead to cellular transformation. Overall, however, the LPV large T antigen is structurally more similar to the PyV large T antigen, a protein which does not bind to p53 (50). Studies with SV40 T-antigen fragments indicate that the residues important for p53 binding are located within amino acids 271 to 517 (33, 44). Computer analyses (10) show amino acids 335 to 579 of LPV T antigen to be 50.4% identical and 68.6% similar to this putative p53-binding region (Fig. 6). PyV T antigen contains a region which is also 50% identical to the p53-binding region of SV40 T antigen and is 53% identical to the comparable region of LPV T antigen (Fig. 6). The ability of LPV T antigen to complex p53 therefore could not be directly predicted from primary sequence comparisons. Here we present experimental evidence that, like SV40 T antigen, LPV T antigen binds to the p53 protein, leading to its stabilization.

LPV T antigen is complexed to p53 in both tumor-derived cells and cells transformed in culture. The p53 gene is thought to function as a negative regulator of cell growth. When cotransfected with certain oncogenes into rodent cells, the wild-type p53 gene reduces transformation efficiencies (12, 14), and it suppresses the growth of SV40-transformed hamster cells (32) and human colon carcinoma cells in culture (2). Furthermore, the p53 gene is often mutated or

lost in human tumors, supporting its role as a tumor suppressor (for reviews, see references 22, 26–28, and 30). Although the p53 protein was first identified through its interaction with the SV40 large T antigen (23, 29), it has since been shown to complex other viral proteins, including the adenovirus type 5 E1B 55-kDa protein (41) and the human papillomavirus types 16 and 18 E6 proteins (26, 51). Interaction with the SV40 and adenovirus proteins results in the stabilization of p53 (see below), whereas interaction with HPV E6 appears to lead to the degradation of p53 (43). Complex formation with p53 and the degradation of the p53 protein may represent two different mechanisms, both leading to the inactivation of growth suppression, or these events may have different roles in transformation. The actual biological consequences of the interactions between viral proteins and p53 remain unknown.

Using coprecipitation assays, we demonstrated that the LPV T antigen also forms a stable complex with p53. In all cell lines tested, LPV T antigen was recovered with two independent monoclonal antibodies that recognize different p53 epitopes. Increased p53 levels characteristic of SV40 T antigen-transformed cells were observed. A third prominent protein of 68 kDa was also present in immunoprecipitations from extracts of LCP726, a tumor-derived cell line. Antibodies specific for hsp70 coprecipitated both p53 and LPV T antigen. Previous studies have shown that hsp70 binds to mutant forms of p53 (20, 22, 28, 37) and to SV40 T antigen in independent complexes (31, 42). Our analysis could not

FIG. 5. p53 in LPV-transformed cells is in the wild-type conformation. All cell lines were incubated with 35 S-labeled amino acids for 1.5 h. p53 was first immunoprecipitated from extracts with either PAb421 or PAb246. The supernatants from BALB/c 3T3, M53-1, and LCP726 cells were incubated again with the same antibody. After the second precipitation, the resulting supernatant was divided into two equal portions, and each portion was incubated with either PAb421 or PAb246. The SLT/B5 extract was first cleared with one of the anti-p53 antibodies, and the resulting supernatant was then divided and immunoprecipitated as above. (A) The controls, BALB/c 3T3 nontransformed cells (lanes 1 to 8) and M53-1 cells which express mutant p53 protein (lanes 9 to 16). (B) Analyses of LCP726 (lanes 1 to 8) and SLT/B5 (lanes 9 to 15) cell extracts. All samples were analyzed on a single gel. The positions of the molecular mass markers (in kilodaltons) and of the p53 protein in each extract are indicated.

distinguish whether the hsp70 protein was part of a complex with both LPV T antigen and p53 or was independently associated with each protein. Since the 68-kDa protein was not detectable in coprecipitations from the BALB/c 3T3 cells transformed with LPV T antigen, further studies are required to determine the significance of the simultaneous association of hsp70 with LPV T antigen and p53 in the LCP726 cell line.

Complex formation with LPV T antigen extends the half-life of p53. Elevated levels of p53 relative to the amount found in BALB/c 3T3 cells were detected in all cell lines transformed with the LPV T antigen. Moreover, pulse-chase experiments indicated that the half-life of p53 in all cases increased 9- to 22-fold over the 19-min half-life of the protein in BALB/c 3T3 cells. In these experiments, the SV40 T antigen extended the p53 half-life approximately 27-fold. The half-life of LPV T antigen was much shorter than that of SV40 T antigen (5 to 9 h compared with >24 h, respectively) which may contribute to the somewhat shorter p53 half-life in cells transformed by LPV T antigen. Also, in the LPV-transformed BALB/c 3T3 cells, submolar amounts of the LPV T antigen with respect to levels of p53 were present in the complex. We observed the same phenomenon in all six

FIG. 6. Alignment of amino acids from the large T antigens of SV40, LPV, and mouse PyV that are identical to the p53-binding region of SV40 large T antigen, using a Genetics Computer Group sequence analysis program (10). A line between amino acids indicates identity, and dots represent conservative changes.

isolates tested (data are shown for the SLT/B5 and SLT/B56 lines). Perhaps when LPV T antigen is present in limiting amounts, the complexed p53 forms oligomers (21). Because the LPV antiserum detects p53 (albeit inefficiently) in BALB/c cells, we could not determine unambiguously whether a stable population of p53 existed which was not complexed to LPV T antigen. Further analysis of the LPV T antigen-p53 complex will be required to determine the exact nature of the physical association between the two proteins.

Although stabilization of p53 was observed in several independently derived LPV-transformed cell lines, it could have resulted from fortuitous mutation of the p53 gene. Several different single-amino-acid changes in p53 result in the loss of its growth suppression phenotype and in the simultaneous activation of its ability to cooperate with oncogenes such as *v-ras* in the transformation of cultured primary cells (22, 26-28). These mutations also lead to a 2- to 12-fold increase in the stability of p53 (22, 26-28). Our results from sequential immunoprecipitation with PAb246 (which recognizes wild-type p53; see Results) and PAb421 (which recognizes all p53 forms) indicate that the only detectable p53 in all of the LPV-transformed cell lines analyzed was wild type (PAb246⁺). Since this assay is interpreted on the basis of a previous correlation, we cannot exclude the possibility that a novel PAb246⁺ form of mutant p53 is present. However, this explanation is unlikely, given the number of independent isolates that were examined.

In summary, we have demonstrated here that wild-type p53 is complexed to LPV T antigen in both an LPV-induced choroid plexus tumor cell line and BALB/c 3T3 cells transformed by LPV T antigen in culture. Moreover, p53 complexed to LPV T antigen is stabilized 9- to 22-fold more than

is the p53 in BALB/c 3T3 cells. Our studies indicate that LPV T antigen is more related to the SV40 class of large T antigens than to the PyV class with respect to transformation-related activities. These observations further suggest that p53 may also be a cellular target in LPV T-antigen-mediated transformation and tumorigenesis. Further comparison among the tumor virus proteins and correlation between their common transforming abilities and biochemical properties should provide additional insight into the mechanisms of cell growth control.

ACKNOWLEDGMENTS

We thank Jim Pipas for valuable discussion and for critical reading of the manuscript. We thank Ashok Srinivasan for providing his cell line and for his expert advice on cell culture transformation. Don DeFranco and Wolfgang Deppert were both very helpful in supplying their immunological reagents for our studies. The technical support of Crystal Petrone, Tricia Ambroziak, and Ken McGaffin and the photographic talent of Kimberly Pasko are greatly appreciated.

This work was supported by Public Health Service grant CA-46283 from the National Cancer Institute and by a grant from the Pittsburgh Supercomputing Center.

REFERENCES

1. Aaronson, S. A., and G. Todaro. 1968. Development of 3T3-like lines from mouse embryo cultures. *J. Cell Physiol.* **42**:141-148.
2. Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Willson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science* **249**:912-915.
3. Brade, L., W. Vogel, L. Gissman, and H. zur Hausen. 1981. Propagation of B-lymphotropic papovavirus (LPV) in human B-lymphoma cells and characterization of its DNA. *Virology* **114**:228-235.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**:248-254.
5. Brinster, R. L., H. Y. Chen, A. Messing, T. Van Dyke, A. J. Levine, and R. D. Palmiter. 1984. Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. *Cell* **37**:367-379.
6. Chen, J., K. Neilson, and T. Van Dyke. 1989. Lymphotropic papovavirus early region is specifically regulated in transgenic mice and efficiently induces neoplasia. *J. Virol.* **63**:2204-2214.
7. Chen, J., and T. Van Dyke. Submitted for publication.
8. Chen, S., and E. Paucha. 1990. Identification of a region of simian virus 40 large T-antigen required for cell transformation. *J. Virol.* **64**:3350-3357.
9. DeCaprio, J. A., J. W. Ludlow, J. Figge, J.-Y. Shew, C.-M. Huang, W.-H. Lee, E. Marsilio, E. Paucha, and D. M. Livingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.
10. Devereux, J., P. Haerberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387-395.
11. Dyson, N., R. Bernards, S. H. Friend, L. R. Gooding, J. A. Hassell, E. O. Major, J. M. Pipas, T. Van Dyke, and E. Harlow. 1990. Large T antigens of many polyomaviruses are able to form complexes with the retinoblastoma protein. *J. Virol.* **64**:1353-1356.
12. Eliyahu, D., D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, and M. Oren. 1989. Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc. Natl. Acad. Sci. USA* **86**:8763-8767.
13. Finlay, C. A., P. W. Hinds, T.-H. Tan, D. Eliyahu, M. Oren, and A. J. Levine. 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* **8**:531-539.
14. Finlay, C. A., W. Hinds, and A. J. Levine. 1989. The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**:1083-1093.
15. Furuno, A., T. Kanda, and K. Yoshiiki. 1986. Monkey B-lymphotropic papovavirus genome: the entire DNA sequence and variable regions. *J. Med. Sci. Biol.* **39**:151-161.
16. Gannon, J. V., R. Greaves, R. Iggo, and D. P. Lane. 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *EMBO J.* **9**:1595-1602.
17. Gurney, E. G., R. O. Harrison, and J. Fenno. 1980. Monoclonal antibodies against simian virus 40 T-antigens: evidence for distinct subclasses of large T antigen and for similarities among nonviral T antigens. *J. Virol.* **34**:752-763.
18. Hanahan, D. 1989. Transgenic mice as probes into complex systems. *Science* **246**:1265-1275.
19. Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* **39**:861-869.
20. Hinds, P. W., C. A. Finlay, A. B. Frey, and A. J. Levine. 1987. Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines. *Mol. Cell. Biol.* **7**:2863-2869.
21. Kraiss, S., A. Quaiser, M. Oren, and M. Montenarh. 1988. Oligomerization of oncoprotein p53. *J. Virol.* **62**:4737-4744.
22. Lane, D. P., and S. Benchimol. 1990. p53: oncogene or anti-oncogene? *Genes Dev.* **4**:1-8.
23. Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (London)* **278**:261-263.
24. Lanford, R. E., and J. S. Butel. 1979. Antigenic relationship of SV40 early proteins to purified large T polypeptide. *Virology* **97**:295-306.
25. Larose, A., N. Dyson, M. Sullivan, E. Harlow, and M. Bastin. 1991. Polyomavirus large T mutants affected in retinoblastoma protein binding are defective in immortalization. *J. Virol.* **65**:2308-2313.
26. Levine, A. J. 1990. The p53 protein and its interactions with the oncogenic products of the small DNA tumor viruses. *Virology* **177**:419-426.
27. Levine, A. J., and J. Momand. 1990. Tumor suppressor genes: the p53 and retinoblastoma sensitivity genes and gene products. *Biochim. Biophys. Acta* **1032**:119-136.
28. Levine, A. J., J. Momand, and C. A. Finlay. 1991. The p53 tumour suppressor gene. *Nature (London)* **351**:453-456.
29. Linzer, D. I. H., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* **17**:43-52.
30. Marshall, C. J. 1991. Tumor suppressor genes. *Cell* **64**:313-326.
31. May, E., C. Breugnot, A. Duthu, and P. May. 1991. Immunological evidence for the association between simian virus 40 115-kDa super T antigen and hsp70 proteins in rat, monkey, and human cells. *Virology* **180**:285-293.
32. Mercer, W. E., M. Amin, G. J. Sauve, E. Appella, S. J. Ullrich, and J. W. Romano. 1990. Wild type human p53 is antiproliferative in SV40-transformed hamster cells. *Oncogene* **5**:973-980.
33. Michalovitz, D., L. Fischer-Fantuzzi, C. Vesco, J. M. Pipas, and M. Oren. 1987. Activated Ha-ras can cooperate with defective simian virus 40 in the transformation of nonestablished rat embryo fibroblasts. *J. Virol.* **61**:2648-2654.
34. Monier, R. 1986. Transformation by SV40 and polyoma, p. 247-294. In N. P. Salzman (ed.), *The Papovaviridae*. Plenum Press, New York.
35. Oren, M., W. Maltzman, and A. J. Levine. 1981. Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Mol. Cell. Biol.* **1**:101-110.
36. Pawlita, M., A. Clad, and H. zur Hausen. 1985. Complete DNA sequence of lymphotropic papovavirus: prototype of a new species of the polyomavirus genus. *Virology* **143**:196-211.
37. Pinhasi-Kimhi, O., D. Michalovitz, A. Ben-Zeev, and M. Oren. 1986. Specific interaction between the P53 cellular tumour antigen and major heat shock proteins. *Nature (London)* **320**:182-185.
38. Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin. 1982. The roles of individual polyoma

- virus early proteins in oncogenic transformation. *Virology* **300**: 713–718.
39. Reich, N. C., and A. J. Levine. 1984. Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. *Nature* (London) **308**:199–201.
40. Reihnsaus, E., M. Kohler, S. Kraiss, M. Oren, and M. Montanari. 1990. Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. *Oncogene* **5**:137–145.
41. Sarnow, P., Y. S. Ho, J. Williams, and A. J. Levine. 1982. Adenovirus E1b-58kD tumor antigen and SV40 large T-antigen are physically associated with the same 54kD cellular protein in transformed cells. *Cell* **28**:387–394.
42. Sawai, E. T., and J. S. Butel. 1989. Association of a cellular heat shock protein with simian virus 40 large T antigen in transformed cells. *J. Virol.* **63**:3961–3973.
43. Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**:1129–1136.
44. Schmieg, F. I., and D. T. Simmons. 1988. Characterization of the in vitro interaction between SV40 T antigen and p53: mapping the p53 binding site. *Virology* **164**:132–140.
45. Springer, T. A. 1989. Purification of proteins by precipitation, p. 10.16.1–10.16.11. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. Greene Publishing Assoc. and Wiley Interscience, New York.
46. Srinivasan, A., K. W. C. Peden, and J. M. Pipas. 1989. The large tumor antigen of simian virus 40 encodes at least two distinct transforming functions. *J. Virol.* **63**:5459–5463.
47. Thompson, D. L., D. Kalderon, A. E. Smith, and M. J. Tevethia. 1990. Dissociation of Rb-binding and anchorage-independent growth from immortalization and tumorigenicity using SV40 mutants producing N-terminally truncated large T antigens. *Virology* **178**:15–34.
48. Tooze, J. 1981. DNA tumor viruses. *Molecular biology of tumor viruses*, 2nd ed., revised. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. Wade-Evans, A., and J. R. Jenkins. 1985. Precise epitope mapping of the murine transformation-associated protein, p53. *EMBO J.* **4**:699–706.
50. Wang, E., P. N. Friedman, and C. Prives. 1989. The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. *Cell* **57**:379–392.
51. Werness, B. A., A. J. Levine, and P. M. Howley. 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**:76–79.
52. Yewdell, J. W., J. V. Gannon, and D. P. Lane. 1986. Monoclonal antibody analysis of p53 expression in normal and transformed cells. *J. Virol.* **59**:444–452.
53. zur Hausen, H., and L. Gissman. 1979. Lymphotropic papovavirus isolated from African green monkey and human cells. *Med. Microbiol. Immunol.* **167**:137–153.