The SV40 Large T Antigen-p53 Complexes Bind and Activate the Insulin-like Growth Factor-I Promoter Stimulating Cell Growth

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Abstract

Inactivation of cellular p53 is a crucial step in carcinogenesis. Accordingly, p53 is inactivated in most human cancers by different mechanisms. In cells infected with DNA tumor viruses, p53 is bound to the viral tumor antigens (Tag). The current “dogma” views the Tag-p53 complexes as a way of sequestering and inactivating p53. Using primary human cells and SV40-transformed human cells, we show that in addition to inactivating p53 tumor suppressor activities, the Tag-p53 complex has growth stimulatory activities that are required for malignant cell growth. We found that in human cells, Tag-p53 complexes regulate transcription of the insulin-like growth factor I (IGF-I) gene by binding to the IGF-I promoter together with pRB and p300. Depletion of p53 leads to structural rearrangements of this multiprotein complex, resulting in IGF-I promoter transcriptional repression and growth arrest. Our data provide a novel mechanistic and biological interpretation of the p53-Tag complexes and of DNA tumor virus transformation in general. In the model we propose, p53 is not a passive inactive partner of Tag. Instead the p53-Tag complex promotes malignant cell growth through its ability to activate the IGF-I signaling pathway. [Cancer Res 2008;68(4):1022–9]

Introduction

The p53 protein plays a critical role in carcinogenesis. Malignant transformation requires that p53 becomes inactivated to prevent p53-mediated apoptosis or cell growth arrest in cells that have genetic damage. Inactivation of p53 impairs DNA repair, thus favoring the early steps of carcinogenesis. The latter effect is mediated mostly by the ability of p53 to induce p21 expression, a cyclin-dependent kinase inhibitor that in turn causes cell cycle arrest, allowing DNA repair to take place. By inducing p21 expression, p53 prevents cells that have accumulated genetic damage from undergoing mitosis and propagating the damaged DNA to the descendants. Because of its critical role in regulating proper cell growth, “normal” wild-type p53 activity is undesirable for cancer cells. Accordingly, p53 must be inactivated to “create” human tumor cells in the laboratory (1). Moreover, the p53 pathway is found to be inactivated in most human tumors either by direct mutation or by alterations of p53 partners. DNA tumor viruses target p53 through the activities of their tumor antigens (the large T antigen, or Tag, of human polyomaviruses JCV and BKV, the SV40 Tag, the E1b of adenoviruses), which bind to and inactivate p53 tumor suppressor functions (2). Some viruses have developed additional strategies for inactivating p53: for example, the human papillomavirus 16 (HPV16) oncoprotein E6 binds to cellular p53, promoting its ubiquitylation and degradation (3, 4).

The current hypothesis is that, on Tag binding, p53 loses its ability to work as a transcription factor and as a tumor suppressor gene (5–7).

Unexpectedly, some studies have shown that p53 complexes to Tag can still bind DNA at p53 binding sites (8–10). Moreover, Tag-bound p53 extracted from monkey and human cells was able to stimulate transcription of a p53-regulated promoter in cell-free extracts (9). SV40-mediated transformation of fibroblasts was enhanced by wild-type mouse p53 (11). Similarly, transformation of rat fibroblasts required both Tag and a metabolically stabilized p53 (12). Animal studies have shown that SV40 is more efficient in promoting tumor growth in the presence of wild-type p53 (13). These data did not fit with the generally accepted hypothesis that polyomavirus Tags bind to and inhibit cellular p53. Here we investigated the possible biological effects of Tag-p53 complexes on cell growth and transformation. We focused our studies on the insulin-like growth factor-I (IGF-I) pathway because this pathway has a critical role in regulating normal and malignant cell growth and because SV40-mediated transformation is dependent on the IGF-I signaling pathway (14–16).

Materials and Methods

Plasmids, oligonucleotides, and antibodies are described in Supplementary data.

Cells and gene transfer procedures. Primary human mesothelial cell cultures were obtained from nontumorous donors, cultured, and characterized as described (17). These cells contain wild-type p53 (17). SV40-transformed human mesothelial cells (S-HML) were obtained through human mesothelial cell infection with SV40 virions (10 plaque-forming units/cell). Six to eight weeks after infection, tridimensional foci of transformed cells were handpicked and cultured into cell lines. The latter were grown in DMEM supplemented with 5% fetal bovine serum (FBS). In this study, we confirmed critical results in three independent S-HML. Primary human astrocytes were from Lonza and were cultured as recommended. SV40-transformed astrocytes were obtained and characterized after SV40 infection of primary astrocyte cultures essentially as previously described for S-HML (17). Retroviral packaging was done using 293 human kidney cells following standard procedures. S-HML transduced with the retrovirus expressing the tetracycline regulator (TR; TET-ON system, Clontech Laboratories, Inc.) were selected with 600 µg/mL G418. After selection, the functionality of the system was assayed as recommended by the manufacturer. These cells were transduced with the retrovirus expressing HPV16 E6 (in the presence of doxycycline). Transduced cells were then selected with 600 µg/mL hygromycin and the resulting clone was named S-HML/E6.
expressed E6 transiently through electroporation. Transfection was done with an electroporator (Gene Pulser II, Bio-Rad) using the following parameters: 300 kV and 975-mC pulse capacity; 1 μg of plasmid DNA per 10^6 cells. Efficiency of transfection was >95%. The level of p53 down-regulation was measured 24 h after transfection.

**Results**

S-HML/E6 cells expressed functionally active recombinant HPV16 E6. We wanted to study the effects of lowering p53 in cells

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**Figure 1.** E6 induction down-regulates p53 and p53-regulated gene products. A, expression of Tag and p53 in S-HML expressing E6. B, reduced p53 expression is paralleled by decreased p21 and mdm2 expression. Cells were treated with or without doxycycline (DOX) for 48 h. E6, S-HML/E6 cells. Cont, control cells. C, transient transfection of S-HML with pE6CDNA4A causes marked p53 down-regulation. C24, control 24 h after transfection; E624, E6 24 h after transfection; C48, control 48 h after transfection; E648, E6 48 h after transfection.
expressing the SV40 Tag. To achieve this goal, we expressed HPV16 E6 in S-HML. We used a stable, tetracycline-inducible transduced cell clone expressing a fusion protein consisting of six histidines at its NH2-terminal portion (for conjugation to Ni-charged carriers), an Xpress peptide flag, and the full-length HPV16 E6 (see Supplementary data). These cells (named S-HML/E6) express the SV40 Tag and, on doxycycline treatment, also express E6 that bound and degraded p53 (Fig. 1; Supplementary Fig. S1).

**Decreased amounts of p53 in S-HML/E6 correspond to decreased expression of proteins transcriptionally regulated by p53 and cause cell growth arrest.** We measured p53 and Tag expression levels at different time points after doxycycline induction in S-HML/E6 cells. Forty-eight hours after doxycycline-mediated induction of HPV16 E6, S-HML/E6 had reduced expression of p53 and p21 and mdm2 (proteins regulated by p53; reviewed in ref. 19); Tag expression was not influenced (Fig. 1A and B). We hypothesized that these effects could have caused apoptosis/mitotic catastrophe or a proliferative advantage. Instead, annexin V/propidium iodide staining followed by fluorescence-activated cell sorting (FACS) analysis did not show evidence of apoptosis or the appearance of aberrant DNA peaks (an indication of mitotic catastrophe), and S-HML/E6 showed a doxycycline dose-dependent reduction in DNA synthesis compared with controls (Fig. 2A). Because doxycycline has pleiotropic effects that might have influenced these findings, we expressed recombinant E6 protein in transiently transfected S-HML. We obtained identical results: E6-transfected S-HML had undetectable p53 and were growth arrested compared with controls (Figs. 1C and 2B). The reciprocal experiments (growth curves for the inducible system and DNA incorporation assay for S-HML transiently transfected with E6) gave identical results (Supplementary Fig. S2). To study why depletion of cellular p53 in S-HML resulted in growth arrest, we investigated genes that are transactivated by Tag (20, 21). We found that doxycycline treatment of S-HML/E6 abolished IGF-I precursor expression and the IGF-I receptor (IGF-IR; Fig. 2C). The effects observed on E6 transfection seemed to be dependent on the presence of Tag because E6 transfection of primary human mesothelial cells (which do not contain SV40) caused the opposite effect: a 4.3-fold increase of IGF-I expression (Fig. 2D, top). Because E6 did not influence Tag expression but influenced p53 expression

![Figure 2](image-url)
in S-HML (Fig. 1A), we speculated that the E6 activities in Tag-containing cells were mediated through the degradation of p53. To test our hypothesis that the decreased expression of p21, mdm2, IGF-I, and IGF-IR on E6 induction was related to p53 down-regulation, we deregulated p53 in S-HML using a short hairpin RNA against p53, a dominant negative p53 (p53mt135, which interferes with proper p53 complex formation; Supplementary Fig. S3A and B, respectively), or by overexpressing mdm2 in S-HML (Fig. 2D, bottom); all these approaches yielded reproducible p21, IGF-I, and IGF-IR down-regulation. The most effective and reproducible way to down-regulate p53 expression in S-HML was expressing HPV16 E6 in these cells (Fig. 1C). These results together suggested that the decreased expression of p21, IGF-I, and IGF-IR was related to p53 depletion independently of how that was achieved.

E6-mediated p53 down-regulation causes S-HML cell growth arrest through the IGF-I/IGF-IR signaling pathway. To confirm that p53 depletion in S-HML causes cell growth arrest through IGF-I/IGF-IR signaling, we designed the experiment summarized in Fig. 3A. S-HML were transfected with a plasmid expressing the IGF-IR under the control of a cytomegalovirus (CMV) promoter (1R cells). S-HML transfected with the empty plasmid served as control (1C cells). Both 1R and 1C cells were transfected in parallel either with the E6-expressing plasmid (yielding either 1R6 or 1C6 cells) or with the control plasmid for E6 (yielding either 1Rc or 1Cc cells). Twenty-four hours after transient transfection, cells were cultured in 1% FBS or 1% FBS supplemented with 5 nmol/L purified IGF-I. Forty-eight hours after transient transfection, all cells were analyzed for DNA synthesis by BrdUrd incorporation assay/FACS analyses (Fig. 3B–D). Both 1Rc and 1Cc (these cells do not express E6 and have normal p53 amounts) were able to resume DNA synthesis after IGF-I treatment (Fig. 3B). Instead, 1C6 cells (which have down-regulated p53 and the IGF-IR is under the control of its own promoter) could not resume DNA synthesis (Fig. 3C). However, 1R6 cells (which have down-regulated p53 but an IGF-IR under the control of a CMV promoter; Supplementary Fig. S4C) resumed DNA synthesis on IGF-I stimulation (Fig. 3D). These data confirmed that E6-mediated down-regulation of p53 in S-HML impaired DNA synthesis and that the growth impairment was mediated through effects of the Tag-p53 complexes on the IGF-I signaling pathway. This interpretation was supported by the finding that E6 expression in primary mesothelial cells that do not contain Tag did not affect the rate of DNA synthesis in these cells (Supplementary Fig. S5). We found that down-regulation of p53 with short hairpin RNA in SV40 transformed primary human astrocytes caused down-regulation of the IGF-I precursor and of the IGF-IR (Supplementary Fig. S6), suggesting that the data we observed in human mesothelial cells are of general relevance to human cells.
E6-mediated down-regulation of p53 causes transcriptional repression of p53-regulated promoters and of the IGF-I promoter in S-HML. We hypothesized that the decreased expression levels of p21, mdm2, and IGF-I protein detected after E6-mediated p53 depletion in S-HML could have been mediated at the transcriptional level. Treatment of S-HML with IGF-I caused increased expression of the IGF-IR, whereas a small interfering RNA directed against IGF-I caused decreased IGF-IR expression in S-HML (Supplementary Fig. S7). Therefore, we concluded that the study of the IGF-I promoter regulation would have provided major insights into the positive IGF-I/IGF-IR autocrine feedback loop in S-HML. We used real-time PCR to study the expression of IGF-I and of genes under p53 regulation. We found that E6-mediated p53 down-regulation in S-HML almost abolished p21 and IGF-I mRNA expression and caused 50% and 80% reductions in the expression of Bax (a protein also regulated by p53) and mdm2 mRNAs, respectively (Fig. 4A). Nuclear run-on experiments confirmed that the transcriptional activity of these promoters is suppressed in S-HML expressing E6 (Fig. 4B). To further verify that E6 induction in S-HML caused repressed transcription at the IGF-I promoter, we carried out reporter assays using a plasmid kindly provided by Dr. R. Baserga (Thomas Jefferson University, Philadelphia, PA), in which firefly luciferase was under the control of the rat IGF-I promoter (pSmaBgl-LUC0). Cells were transfected, then cultured in the presence or absence of doxycycline. Forty-eight hours after culturing, 10 μg of total cell lysates were assayed for luciferase activity. The values were measured as firefly luciferase units/renilla luciferase light units. The histogram is the average of 12 independent experiments. The results were as follows: control cells cultured in the absence of doxycycline, 97.89 ± 30.53; control cells cultured in the presence of 2 μg/mL doxycycline, 95.79 ± 34.73; S-HML/E6 cultured in the absence of doxycycline, 83.16 ± 28.42; and S-HML/E6 cultured in the presence of 2 μg/mL doxycycline, 26.32 ± 16.21. *, P > 0.01.

E6 induction in S-HML causes transcriptional repression at p53-regulated promoters. Tag and p53 bind to the rat IGF-I promoter in vitro. A, steady-state levels of the Bax, p21, mdm2, and IGF-I mRNAs in S-HML transfected with a control plasmid (C) and with a plasmid expressing recombinant E6 (E6). The measurements were taken 48 h after transfection. The histogram represents the average of four independent experiments; each measurement was taken in triplicate over a curve of six dilutions of cDNAs. GAPDH mRNA was used as the internal control for each sample. Setting the control plasmid-transfected S-HML at 100%, the E6-transfected S-HML displayed the following averages: Bax, 20.59 ± 14.71%; p21, 0.32 ± 0.04%; mdm2, 53.75 ± 11.25%; and IGF-I, 0.02 ± 0.01%. B, left, representative nuclear run-on experiment done on nuclei of S-HML transfected with either the control plasmid or the E6-expressing plasmid. The experiments were conducted 48 h after transfection. Radioactivity associated with each band was measured with a phosphorimager. Right, average of four independent experiments. Setting the controls at 100%, the E6-transfected S-HML displayed the following averages: p21, 19.84 ± 12.70%; IGF-I, 23.80 ± 17.48%; Bax, 26.98 ± 20.63%; 18S, 92.06 ± 12.70%. Bars, SD. C, reporter assay conducted on S-HML/E6 and control cells in the presence and absence of doxycycline. Firefly luciferase was under the control of the rat IGF-I promoter (pSmaBgl-LUC0). Cells were transfected, then cultured in the presence or absence of antibiotic. Forty-eight hours after culturing, 10 μg of total cell lysates were assayed for luciferase activity. The values were measured as firefly luciferase units/renilla luciferase light units. The histogram is the average of 12 independent experiments. The results were as follows: control cells cultured in the absence of doxycycline, 97.89 ± 30.53; control cells cultured in the presence of 2 μg/mL doxycycline, 95.79 ± 34.73; S-HML/E6 cultured in the absence of doxycycline, 83.16 ± 28.42; and S-HML/E6 cultured in the presence of 2 μg/mL doxycycline, 26.32 ± 16.21. *, P > 0.01.
that showed the highest homology with the rat promoter (72% nucleotide identity) was the one spanning positions −1,952 to −322 of the human IGF-I promoter. This promoter region was analyzed by chromatin immunoprecipitation assay dividing the 1,630-bp DNA promoter region in seven partially overlapping amplicons (Fig. 5A). As a negative control, we used a 266-bp region on chromosome 12q12 (see Supplementary data for a detailed description of this amplicon). We compared nontransfected S-HML with p53-depleted S-HML via E6 transfection. Cells were cross-linked, then cell lysates were mechanically sheared and immunoprecipitated with antibodies specific for either Tag or p53 (Fig. 5B, lanes 3 and 4). The cross-linking in immunoprecipitated materials was reversed and PCR amplified with primers that yielded the amplicons outlined in Fig. 5A. In nontransfected S-HML (cells with an active IGF-I promoter), Tag was associated with the “B” region, and p53 was associated with both the “A” and “B” regions (Fig. 5B). Instead, in p53-depleted S-HML, p53 was associated only with the “B” region (Fig. 5C, lanes 7 and 8) and Tag was associated prevalently with the “A” region (Fig. 5C, top, compare lanes 5 and 6), although the signal corresponding to the “B” region persisted (Fig. 5C, lanes 5 and 6, bottom). This indicated that on E6-mediated p53 down-regulation, the Tag-p53 complex on the IGF-I promoter underwent a conformational change, with Tag moving upstream (i.e., to the A region) from the IGF-I starting codon and p53 losing occupancy of the same region. To further investigate the Tag-p53 complex on the IGF-I promoter, we tested the “A” through “G” region of the IGF-I promoter by chromatin immunoprecipitation for two major binding partners of Tag: pRb and p300 (reviewed in ref. 2). When we immunoprecipitated S-HML extracts with a pRb-specific antibody, we PCR amplified region “B” (Fig. 5D, lane 3). When we immunoprecipitated with a p300-specific antibody, we amplified both “A” and “B” regions (Fig. 5D, lane 8). No other region of the portion of the IGF-I promoter was amplified. These data indicated that within the 538 bp encompassing the “A” and “B” regions of the IGF-I promoter, there is a multiprotein complex that includes Tag, p53, pRb, and p300. When we decreased p53 expression through E6 induction, we were still able to amplify region “B” by immunoprecipitating with the pRb-specific antibody (Fig. 5D, lane 7); however, immunoprecipitations conducted with the p300 antibody failed to yield both the “A” (not shown) and “B” amplicons (Fig. 5D, lane 8). This indicated that on p53 depletion, the multiprotein complex at regions −1,952 to −1,414 of the IGF-I promoter underwent modifications that included structural rearrangements of individual complex partners and exit of p300, a transcriptional coactivator (22). The result of these modifications was paralleled by transcriptional inhibition at the IGF-I promoter. We detected no quantitative differences in the amount of pRb bound to the “B” amplicon before and after E6-mediated p53 depletion in S-HML by quantitative PCR (Supplementary Fig. S9B). No E6 association with the IGF-I promoter was detected. Furthermore, no association of either Tag or p53 was detected on the IGF-1R promoter by chromatin immunoprecipitation analysis (not shown).

Discussion

More than 30,000 articles examining the role and function of p53 have appeared in the literature since its discovery in 1979. This extraordinary amount of work on a single gene reflects the central role that p53 has in governing normal cell growth and, consequently, in determining the outcome of genetic insults that may lead to malignant cell transformation and tumor cell growth. In spite of the enormous amount of scientific literature on p53, research on this protein continues to turn up new surprises.

One of the current “dogmas” about p53 is that its transcriptional and biological activities are impaired by its binding to the Tags of...
DNA tumor viruses. A similar dogma in the DNA tumor virus field assumes that the oncogenes of these viruses bind to and “inactivate” cellular p53, a process that is required for viral replication and also for virus-mediated cellular transformation.

Here, we present evidence indicating that this current dogma is only partially correct because newly formed Tag-p53 complexes acquire new transcriptional and biological activities. We found that this complex binds to the IGF-I promoter, stimulating IGF-I expression and the IGF-I signaling pathway, an effect that leads to cell growth. Specifically, we found that the Tag-p53 complexes interact with the IGF-I promoter as part of a complex that consists of several partners, including pRB and p300. When we depleted p53, we observed a loss of p300 on the IGF-I promoter.

Our results provide a rationale to a number of studies that had found that the presence of wild-type p53 was required to stimulate transcription and for polyomaviruses-mediated malignant cell transformation (8–13).

These findings support recent studies showing that Tag requires p53 to interact with p300, and RNAi-mediated p53 depletion disrupts Tag-p300 interactions (23). Therefore, we propose the following model (Fig. 6). In normal S-HML, a multiprotein complex that includes Tag, p53, pRB, and p300 occupies positions −1,952 to −1,414 (approximately) of the IGF-I promoter. On p53 depletion (obtained using different techniques), this complex undergoes structural rearrangements, probably as a result of the exit of some critical components (such as p300) that ultimately regulate the transcription of the IGF-I gene.

The biological effects observed on SV40 infection are species specific (24) because rodent cells are nonpermissive to SV40 replication whereas human cells are. Therefore, animal models could not be used to test in vivo the possible oncogenic effects of the findings reported here.

In our experimental model, we used mesothelial cells and SV40. Specifically, we used different primary (normal) human mesothelial cells obtained from separate donors with nonmalignant pleural effusions (17). We used human mesothelial cells because these cells allow SV40 replication and are rarely lysed by the virus; thus, human mesothelial cells are ideal to study the biological interactions between Tag and p53 (17). Human mesothelial cells are also frequently transformed by SV40 (17, 24), an effect that allowed us to compare the Tag-p53 biological activities in both primary and malignant human cells. However, the results presented here seem to be of general relevance because we observed similar effects in SV40-transformed primary human astrocytes. It should be noted that p53 depletion caused decreased IGF-IR expression in both S-HML and SV40-transformed human astrocytes. Although we did not detect a direct association of either p53 or Tag on the IGF-IR promoter, Tag-p53 complexes may indirectly regulate IGF-IR expression. Alternatively, the IGF-IR may be mainly under a positive feedback loop regulated by autocrine IGF-I. The results presented in Supplementary Fig. S7 seem to support such interpretation.

In summary, our data provide a novel mechanistic and biological interpretation of the p53-Tag complexes and possibly of DNA tumor virus transformation in general. In the model, we propose that the p53-Tag complex in human cells promotes malignant cell growth through its ability to activate the IGF-I signaling pathway. The results presented here suggest that there is a “threshold” effect for p53 when complexed to Tag, and that a correct stoichiometry between these two components influences the biological effects of Tag-p53 complexes.

**Acknowledgments**


Grant support: National Cancer Institute grant R21 CA91122 and American Cancer Society grant RSG-05-077 (M. Bocchetta) and grants RO1CA 90265-01 and P01CA114071-01 (M. Carbone).

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We thank Drs. Antonio Pannuti, Haning Yang, John Jenkins, Premkumar Reddy, and James Pipas for critical reading of the manuscript; Drs. Renato Baserga and Martin W. Kast for providing critical reagents; and Patricia Simms for her invaluable help with FACS analysis.

**References**


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