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The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein

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The observed interaction between p53 and the oncoproteins encoded by several DNA tumor viruses suggests that these viruses mediate their transforming activities at least in part by altering the normal growth regulatory function of p53. In this study we examined the effect of viral oncoprotein expression on the transcriptional transactivation function of wild-type p53 in human cells. Plasmids expressing human p53 were cotransfected with either SV40 large T-antigen or human papillomavirus (HPV) type 16 E6 expression plasmids and assayed for transactivation function using a reporter gene driven by a p53-responsive promoter containing multiple copies of the consensus p53 DNA binding motif, TGCCT. Both large T-antigen and E6 were able to inhibit transactivation by wild-type p53. Furthermore, SV40 T-antigen mutants that are defective for p53 binding were not able to inhibit transactivation and HPV E6 proteins that were either mutant or derived from non-oncogenic HPV types and unable to bind p53, had no effect on p53 transactivation. These results demonstrate the physiological relevance of the interaction of SV40 T-antigen and HPV E6 oncoproteins with p53 *in vivo* and suggest that the transforming functions of these viral oncoproteins may be linked to their ability to inhibit p53-mediated transcriptional activation.

Key words: HPV E6/human papillomavirus/p53/SV40 large T-antigen/transcriptional activation

Introduction

The p53 gene encodes a cellular phosphoprotein that has been associated with both neoplastic transformation and the control of cell growth. The wild-type gene product appears to function as a negative regulator of cell growth and proliferation. In tissue culture experiments overexpression of wild-type p53 results in suppression of the transformed phenotype, and coexpression of p53 has been shown to inactivate the transforming ability of various cellular and viral oncogenes (Eliyahu *et al.*, 1989; Finlay *et al.*, 1989; Hinds *et al.*, 1989). In contrast, deletion or mutation of wild-type p53 can result in a loss of the growth suppressive function and in some cases convert p53 into a dominantly acting oncogene (Eliyahu *et al.*, 1988; Finlay *et al.*, 1989). Mutations within the highly conserved conformational domains of p53 have been observed with high frequency in many different types of human cancers (Baker *et al.*, 1989; Nigro *et al.*, 1989; Takahashi *et al.*, 1989; Mulligan *et al.*, 1990; Bartek *et al.*, 1990; Hollstein *et al.*, 1991) and are often accompanied by loss of the wild-type p53 allele. Mutant p53 alleles have also been detected in families with Li-Fraumeni syndrome, a genetic disorder that results in a predisposition to cancer in the affected individuals (Malkin *et al.*, 1990).

Mutant p53 proteins often exhibit an increased half-life such that high steady state levels of mutant protein accumulate in the cell (Oren *et al.*, 1981) and it has been suggested that some mutant p53 proteins act transdominantly by forming inactive oligomeric complexes with wild-type p53 (Martinez and Levine, 1991; Milner and Metcalf, 1991).

A number of small DNA tumor viruses appear to have evolved mechanisms to inactivate p53 in virus-transformed cells through complex formation. The p53 protein was originally identified through its ability to interact stably with large T-antigen (T-ag) in SV40-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979) and has since been shown to complex with other viral proteins known to play a role in transformation, i.e. the adenovirus type 5 E1B 55 kDa protein (Sarnow *et al.*, 1982) and the E6 proteins of oncogenic human papillomavirus (HPV) types 16 and 18 (Werness *et al.*, 1990). The ability of either SV40 T-ag or the E1B 55 kDa protein to associate physically with p53 has been well established *in vivo* and the interaction of these viral oncoproteins with p53 increases its half-life and steady state level in the cell (Oren *et al.*, 1981; Reich *et al.*, 1983). Although the functional consequences of complex formation between p53 and viral oncoproteins is not well understood, it is presumed that binding to T-ag or E1B inactivates the tumor suppressive function of p53. The ability of HPV types 16 and 18 E6 to complex with p53 has only been clearly demonstrated *in vitro* where the E6-p53 interaction has been shown to result in the selective degradation of p53 via the ubiquitin-dependent proteolytic system (Scheffner *et al.*, 1990; Werness *et al.*, 1990). Thus the observed interactions between viral oncoproteins and p53 suggest that these DNA tumor viruses mediate their transforming activities at least in part by eliminating or altering the normal growth suppressive function of p53.

Although the molecular mechanisms by which p53 exerts its control on cell proliferation are still not known, evidence from a number of laboratories now suggests that p53 can act as a transcriptional activator, perhaps through a direct interaction between p53 and certain transcriptional regulatory elements. p53 is primarily localized in the cell nucleus and the N-terminal 73 amino acids are highly acidic, a feature common to the activating domains of other nuclear proteins known to regulate gene transcription directly (Mitchell and Tjian, 1989). The first demonstration that p53 could function

as a transcriptional activator came from studies where wild-type p53 was fused to the DNA-binding domain of the yeast GAL4 protein. The p53−GAL4 fusion protein was found to activate the expression of a chloramphenicol acetyltransferase (CAT) reporter plasmid containing GAL4 DNA-binding sites adjacent to the promoter sequences (Fields and Jang, 1990; Raycroft *et al.*, 1990; O'Rourke *et al.*, 1990). The transactivation domain of p53 was localized to amino acids 1−42 within the p53−GAL4 fusion construct (Unger *et al.*, 1992). Weintraub and coworkers (1991) provided further evidence that p53 might function as a transcriptional regulator by demonstrating that murine p53 stimulated expression of the muscle-specific creatine kinase (MCK) gene. It was not clear from this study whether p53 potentiates expression by binding directly to a specific DNA sequence element within the MCK promoter or activates transcription indirectly through association with other transcriptional regulatory factors. Specific DNA-binding sequences for wild-type p53 have now been identified both at the SV40 origin of replication and at sites within human genomic DNA (Bargonetti *et al.*, 1991; Kern *et al.*, 1991; Funk *et al.*, 1992) and p53 has recently been shown to activate expression of a target gene that contains multiple copies of the consensus p53 DNA-binding motif defined by Kern *et al.* (1991) linked to a minimal promoter (Funk *et al.*, 1992; Kern *et al.*, 1992; Scharer and Iggo, 1992). Thus wild-type p53 appears to exhibit some of the properties of a sequence-specific transcription factor. Furthermore, several transformation-competent p53 mutants were unable to activate transcription in these assays, providing a strong correlation between the transcriptional activation function of wild-type p53 and its role in the control of cell proliferation (Raycroft *et al.*, 1991; Kern *et al.*, 1992; Unger *et al.*, 1992).

It has been presumed that the normal function of p53 is abrogated as a consequence of its interactions with the transforming proteins of several DNA tumors viruses. The goal of this study was to examine the effects of SV40 large T-ag and HPV-16 E6 protein on the transcriptional transactivation function of wild-type p53.

Results

Wild-type but not mutant p53 functions as a sequence-specific transcriptional activator

In initial studies the transactivation function of p53 had been detected using GAL4−p53 fusion proteins to target p53 to a reporter gene containing GAL4 DNA-binding motifs (Fields and Jang, 1990; Raycroft *et al.*, 1990, 1991; O'Rourke *et al.*, 1990; Unger *et al.*, 1992). Our current study was designed to examine the effect of SV40 T-ag and HPV-16 E6 expression on the transactivation function of p53. For this analysis it was first necessary to construct a target gene that could respond directly to p53 since a chimeric GAL4−p53 fusion protein might be altered with respect to its interaction with the virally encoded oncoproteins. p53 reporter plasmids were constructed by inserting three or six copies of the consensus p53 binding sequence $TGCCTN_{3-5}$ upstream of an enhancerless SV40 promoter linked to the CAT reporter gene (Figure 1a). The target sequences were inserted in either orientation with respect to the SV40 promoter element. The human wild-type p53 cDNA was expressed under the control of the CMV promoter/enhancer. All transfections were performed in H358, a lung cancer-derived cell line which has a homozygous deletion at the p53 locus and does not express endogenous p53 mRNA or protein (Takahashi *et al.*, 1989). The results obtained with this cell line (described below) were confirmed in a second lung cancer cell line, H1299, which is also negative for endogenous p53 expression (Takahashi *et al.*, 1989).

Human wild-type p53 strongly transactivated the test gene constructs (Figure 1b) containing either three or six copies of the consensus binding sequence but had no effect on the pA10CAT control plasmid with only the enhancer-deleted SV40 promoter upstream of CAT. The level of transactivation was 6-fold greater in constructs with six copies of the TGCCT motif than it was with three copies. As reported previously, transactivation of the target gene by p53 was mostly independent of orientation (Kern *et al.*, 1992; Scharer and Iggo, 1992) although in our experiments the

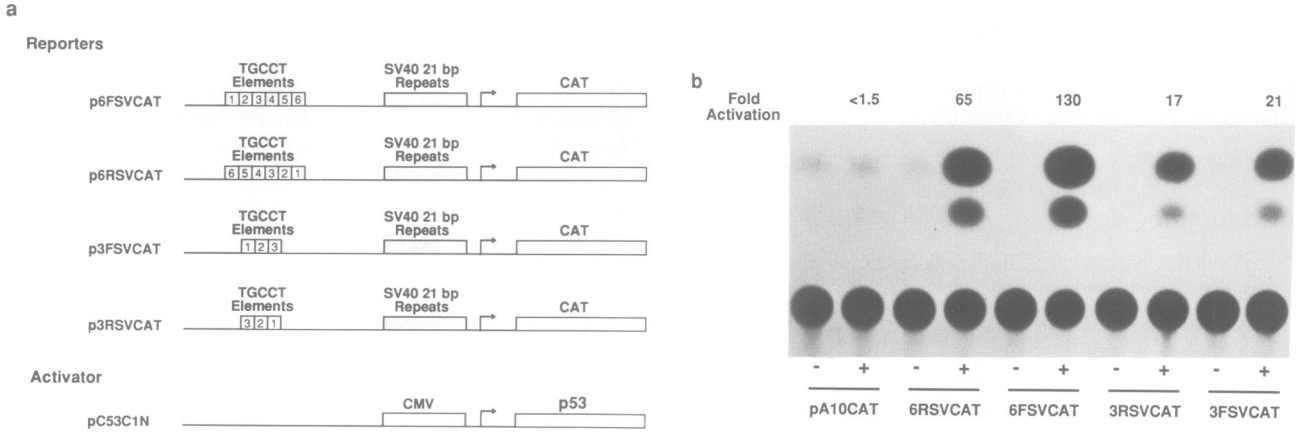

Fig. 1. Human wild-type p53 stimulates expression of a target gene construct containing three or six copies of the consensus p53 binding motif. (**a**) Schematic representation of the reporter gene constructs and the p53 expression plasmid used for cotransfections in H358 cells. The boxes numbered 1−6 or 1−3 in the reporter plasmid refer to the TGCCT motifs within the p53 consensus binding site oligonucleotides 3F and 6F. These constructs include the enhancerless SV40 early region promoter and the CAT gene. pC53C1N consists of the human wild-type p53 cDNA including introns 2−4 under the control of the cytomegalovirus promoter sequences. (**b**) CAT activity produced by transfection of the various reporter plasmids alone (−) or cotransfected with 1 μg pC53C1N (+). The pA10CAT construct contains only the enhancer-deleted SV40 early region promoter sequences linked to the CAT gene. Fold activation represents the percent acetylation produced by cotransfection of the reporter construct (4 μg) and wild-type p53 (1 μg) relative to the CAT activity produced by each construct in the absence of cotransfected p53 expression plasmid. The results represent the average of at least two independent transfections.

forward orientation consistently produced slightly higher levels of CAT expression. Cotransfection of the 6FSVCAT (six copies of TGCCT) with increasing amounts of p53 expression plasmid resulted in a dose-dependent increase in CAT activity. As shown in Figure 2, promoter activity was increased from 2- to 150-fold with half-maximal activity at ~0.01−0.1 μg of cotransfected p53 plasmid. Transfection of >1 μg of p53 expression plasmid only slightly increased the level of transactivation. Even at the highest levels of transfected p53 (5−10 μg pC53C1N), no squelching was observed under these experimental conditions.

In previous studies using GAL4−p53 fusion protein constructs, a number of p53 mutants were compared with wild-type p53 in transactivation assays (Raycroft *et al.*, 1991; Unger *et al.*, 1992). The ability of mutant proteins to activate transcription of reporter gene constructs varied widely. We tested five tumor-derived p53 mutants for the ability to activate transcription of the 6FSVCAT reporter construct when cotransfected into human cells. None of the mutant cDNAs (expressed from the same promoter as wild-type p53) were functional in this assay (Figure 3). In earlier studies using p53−GAL4 fusion proteins, the $p53^{273}$ (Arg → His) mutant (Fields and Jang, 1990) and the $p53^{273}$ (Arg → Leu) mutant (Unger *et al.*, 1992) transactivated at levels equal to or greater than wild-type p53. The $p53^{273}$ (His) mutant was shown to be defective for DNA binding (Kern *et al.*, 1991) and is probably unable to transactivate in the absence of a DNA-targeting peptide such as the GAL4 domain (amino acids 4−147) used in the earlier studies (Fields and Jang, 1990; Unger *et al.*, 1992). Similar results have been reported recently using the same consensus binding site sequence to promote p53-dependent expression in yeast (Scharer and Iggo, 1992) and in another human lung cancer cell line which expressed low levels of endogenous p53 (Kern *et al.*, 1992).

SV40 large T-ag inhibits p53 transactivation but SV40 mutants defective for p53 binding do not

The ability of viral oncoproteins such as SV40 T-ag to function in cell transformation correlates with its ability to interact physically with cellular proteins such as pRb and p53 (Peden *et al.*, 1989; Srinivasan *et al.*, 1989; Zhu *et al.*, 1991) and it has been suggested that complex formation disrupts the normal suppressor function of these cellular proteins. To examine the effect of T-ag expression on the wild-type p53 transactivation function, the H358 lung cancer cell line was cotransfected with wild-type p53, SV40 T-ag expression plasmids and the 6FSVCAT reporter construct. T-ag markedly reduced (>90% reduction) p53-specific transactivation in H358 cells (Figure 4a and b). Furthermore, 5080, an SV40 point mutant which produces a T-ag protein that is unable to bind p53 (Peden *et al.*, 1989), did not affect p53 transactivation. Similarly, deletion mutant pIB147, which encodes a truncated T-ag that does not include the p53-binding domain of large T (Sompayrac and Danna, 1988), was also unable to abrogate transactivation by p53. Although pIB147 has been shown to produce a truncated T-ag that is as stable as the wild-type protein (Sompayrac and Danna, 1988), we were unsure of the stability of the point mutant 5080. Two additional T-ag mutants were therefore analyzed for their effect on p53 transactivation. Mutants 2809 and 2811 contain small linker insertions and encode stable T-ag which fail to bind p53 (Zhu and Cole, 1989; Zhu *et al.*, 1991). Neither mutant significantly inhibited p53 transactivation when compared with the effect observed with the wild-type T-ag construct, pCC2Pa (Figure 5).

The steady state levels of the 5080, 2811 and 2809 full-length T-ag mutants used in this study were examined 36 h after transfection by immunoblot analysis using monoclonal antibody pAb416. The level of T-ag observed for each of the mutants was similar to the wild-type levels observed for PVU-O and pCC2Pa. Thus the ability of SV40 T-ag to inhibit the transcriptional activation function of wild-type p53 was dependent on the ability of T-ag to complex with p53.

To eliminate the possibility that T-ag negatively regulates expression of this CAT reporter construct through interactions with the SV40 promoter sequences, the T-ag coexpression experiment was repeated using a minimal polyoma virus promoter/CAT construct containing multiple

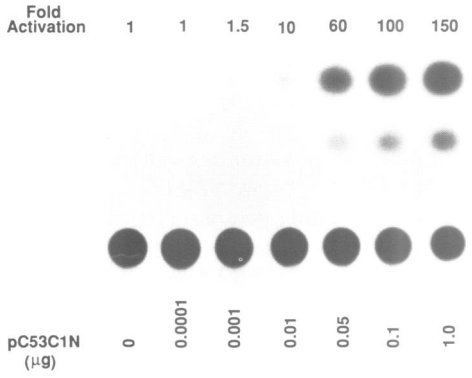

Fig. 2. Dose-dependent stimulation of 6FSVCAT expression by wild-type human p53. H358 cells were cotransfected with 4 μg of the 6FSVCAT reporter construct and increasing amounts of wild-type human p53 expression plasmid (pC53C1N) as indicated. The cells were assayed for CAT activity as described in Materials and methods. A β-galactosidase expression plasmid was included in each transfection to monitor the overall transfection efficiency.

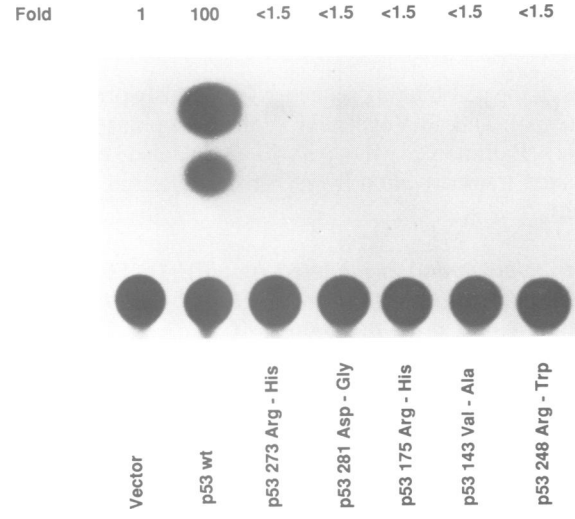

Fig. 3. Transactivation of reporter construct 6FSVCAT by wild-type and various mutant human p53 proteins. H358 lung cells were transfected with 6FSVCAT alone or cotransfected with 6FSVCAT and either wild-type p53 expression plasmid (pC53C1N) or various mutants p53 plasmid constructs as indicated, and assayed for CAT activity 48 h post-transfection. The levels of transactivation results represent the results of at least two independent transfection experiments.

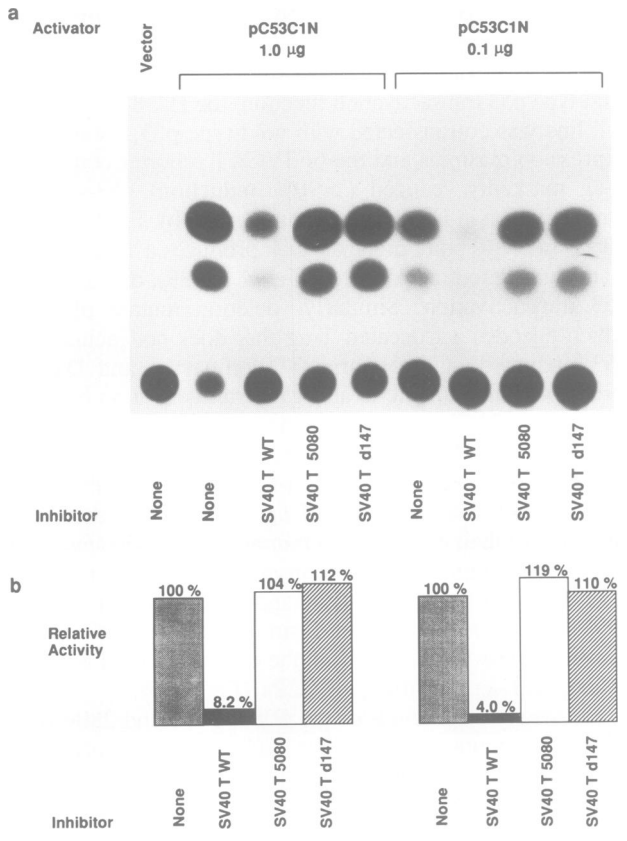

Fig. 4. Inhibition of p53 transactivation by wild-type SV40 large T-ag but not SV40 T-ag mutants 5080 and d147 which are unable to bind p53. (**a**) H358 cells were transfected with 6FSVCAT alone or cotransfected with 4 μg 6FSVCAT, the indicated amounts of pC53C1N and 8 μg of either salmon sperm DNA (None), wild-type SV40 T-ag construct (pPVU-0), SV40 point mutant 5080 (5080) or SV40 deletion mutant d147 (pIB147) and assayed for CAT activity as described in Materials and methods. (**b**) The levels of p53 transactivation in the presence of wild-type SV40 large T-ag and SV40 T-ag mutants. The level of wild-type p53 transactivation (~130-fold) was arbitrarily set at 100%. The levels of activation in the presence of the various SV40 oncoproteins are shown relative to transactivation by p53 alone (no inhibitor).

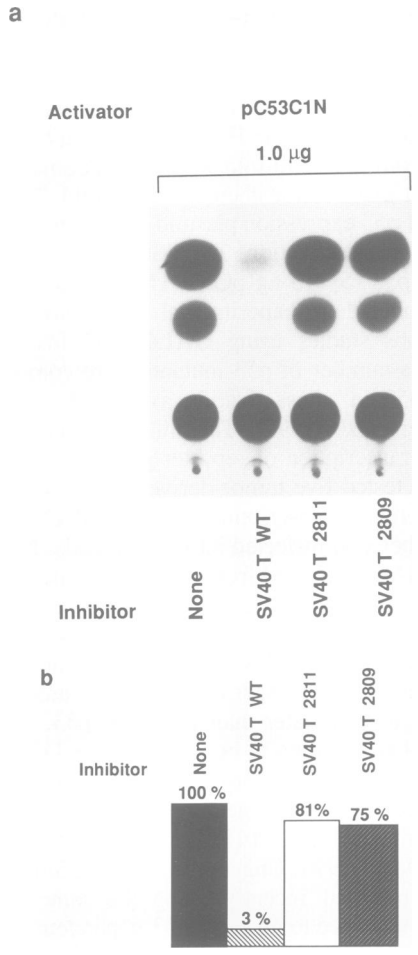

Fig. 5. Inhibition of p53 transactivation by wild-type SV40 large T-ag but not T-ag linker insertion mutants 2809 and 2811 that are unable to bind p53. (**a**) H358 cells were cotransfected with 4μg 6FSVCAT, 1 μg pC53C1N and 8 μg of either salmon sperm DNA (None), wild-type SV40 T-ag construct (pCC2Pa) or SV40 linker insertion mutants 2809 (inA2809) or 2811 (inA2811) and assayed for CAT activity as described in Materials and methods. (**b**) The levels of p53 transactivation in the presence of wild-type SV40 large T-ag and SV40 T-ag mutants. The level of wild-type p53 transactivation was arbitrarily set at 100%. The levels of activation in the presence of the various SV40 oncoproteins are shown relative to transactivation by p53 alone (no inhibitor).

copies of the p53-binding sequence (generously provided by J.Pietenpol and B.Vogelstein, Johns Hopkins Medical School, Baltimore, MD). SV40 T-ag also efficiently abrogated transactivation by p53 in this plasmid (data not shown).

p53 transactivation is inhibited by HPV-16 E6 oncoprotein but not by HPV-11 E6 or by an HPV-16 E6 mutant that does not bind p53

HPV types 16 and 18 are frequently associated with cervical carcinomas and anogenital lesions that are at high risk for malignant progression. HPV types 6 and 11 are considered 'low risk' viruses and are generally associated with benign lesions (zur Hausen and Schneider, 1987). Although the E6 proteins from high risk but not low risk HPV types can complex with wild-type p53 *in vitro* (Werness *et al.*, 1990) and target its proteolytic degradation (Scheffner *et al.*, 1990), the interaction of E6 with p53 *in vivo* has not yet been proven. Low but detectable levels of p53 have been found in a number of HPV-positive cervical carcinoma cell lines consistent with E6 targeting the *in vivo* degradation of p53 but also indicating that not all of the p53 is degraded through interaction with E6 in these cell lines (Scheffner *et al.*, 1991). The assay described in this study provided a method for examining the interaction between HPV E6 and p53 *in vivo* through its effect on p53 transactivation. As shown in Figure 6, a significant decrease (>80% reduction) in the levels of p53 transactivation was observed with HPV-16 E6 but not with HPV-11 E6. These results are consistent with the observed ability of HPV-16 E6 but not HPV-11 E6 to bind detectably to p53. Due to the lack of E6 antibodies that recognize both HPV-16 and HPV-11 E6, we were unable to measure the steady state levels of E6 directly *in vivo*. However, each of the constructs encoded an E6 that was able to transactivate the adenovirus E2 promoter, a function shared by both the high and low risk E6 proteins, indicating that each of these E6 constructs encodes a stable functional protein. JH26 is an HPV-16 E6 mutant with amino acid substitutions at residues 7, 8 and 9. These amino acids are

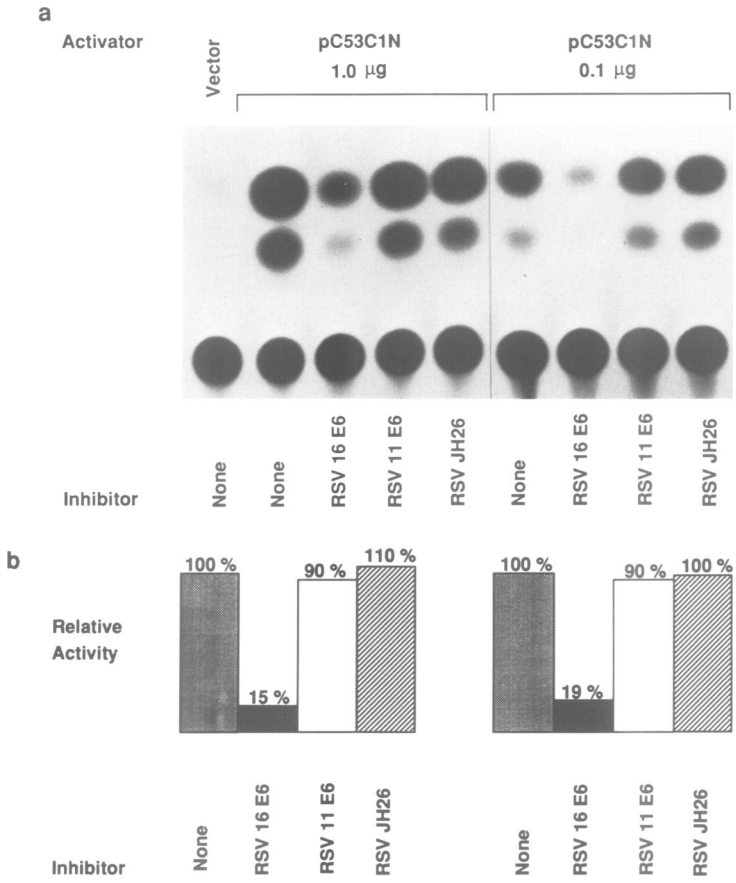

Fig. 6. Inhibition of p53 transactivation by high risk HPV-16 E6 oncoprotein but not low risk HPV-11 E6 or HPV-16 E6 mutant JH26. (**a**) H358 cells were transfected with 6FSVCAT alone or cotransfected with 4 μg 6FSVCAT, the indicated amounts of pC53C1N and 8 μg of either salmon sperm DNA (None), wild-type HPV-16 E6 (p1221), HPV-11 E6 (p2021) or HPV-16 E6 mutant JH26 (p2022) and assayed for CAT activity as described in Materials and methods. (**b**) The levels of p53 transactivation in the presence of wild-type HPV-16 E6, HPV-11 E6 and HPV-16 E6 mutant JH16. The level of wild-type p53 transactivation was arbitrarily set at 100%. The levels of activation in the presence of the various HPV E6 proteins are shown relative to transactivation by p53 alone (no inhibitor).

well conserved among the E6 proteins encoded by different high risk HPV types and were changed to amino acid residues found at these positions in the low risk viruses. The JH26 E6 protein, which is defective for p53 binding *in vitro* (J.Huibregtse, data not shown), was unable to inhibit p53 transactivation. Thus the ability of HPV-16 E6 to decrease the transactivation function of wild-type p53 correlates with its ability to bind p53.

SV40 T-ag and HPV-16 E6 alter the levels of p53 in transiently transfected H358 cells

The ability of SV40 T-ag of HPV-16 E6 to inhibit the transactivation function of p53 is likely to result from the direct interaction of this viral oncoprotein with p53. The association of cellular p53 with SV40 T-ag increases the half-life and steady state level of p53 (Oren *et al.*, 1981) whereas the interaction of HPV-16 E6 with p53 results in the targeted degradation of this cellular protein (Scheffner *et al.*, 1990). In either case the consequence of the p53–viral oncoprotein interaction appears to be the inactivation of p53 and its normal function in the cell. To examine the *in vivo* effect of viral oncoprotein coexpression on the levels of p53 in transiently transfected H358 cells, immunoblot analysis of p53 levels using the monoclonal antibody pAb 1801 was performed 48 h after transfection with a p53 plasmid alone or together with either HPV 16 E6 or SV40 T-ag expression plasmds (Figure 7). Transient coexpression of p53 and T-ag resulted in a dramatically increased level of p53 protein (~10-fold) in H358 cells. In contrast, HPV-16 E6 did not increase the level of p53 but led to a modest but reproducible reduction in the level of p53. Thus in the transient assays performed in this study it appears that both T-ag and the HPV E6 protein affect the levels of cellular p53 in a manner similar to that observed previously in virally transformed cells (Oren *et al.*, 1981; Scheffner *et al.*, 1990, 1991; Band *et al.*, 1991).

Discussion

Wild-type p53 has been shown to function as a suppressor of cell proliferation and transformation. Mutations in p53 have been detected in many human cancers and result in the formation of an altered p53 protein which has lost its tumor suppressor function. The results presented here and the results from other recent studies (Kern *et al.*, 1992; Scharer and Iggo, 1992) demonstrate that wild-type p53 is able to activate gene expression in a sequence-specific manner. In the present study wild-type p53 was able to transactivate efficiently a promoter containing multiple copies of a p53 DNA-binding motif in human cells (Kern *et al.*, 1991) whereas none of five different mutant human p53 proteins were able to function in transactivation. These findings

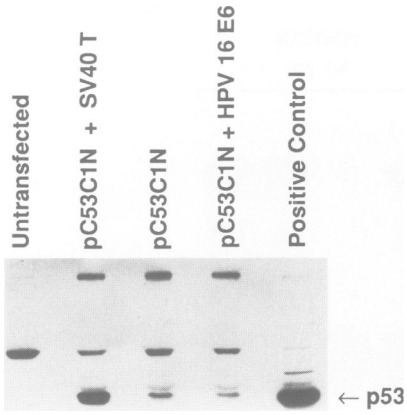

Fig. 7. Immunoblot analysis shows the interaction of p53 and viral oncoprotein products following cotransfection of various expression plasmids into H358 cells. H358 lung cells were cotransfected with 1 μg of pC53C1N and 8 μg of either SV40 large T-ag pPVU-0, HPV-16 E6 (p1221) expression plasmids or salmon sperm DNA. Cell lysates were prepared 48 h after transfection and immunoblotted using p53 antibody pAB1801 as described in Materials and methods. The positive control lysate was prepared from an HPV-negative cervical carcinoma cell line, C-33A, which produces abundant p53 protein (Scheffner *et al.*, 1991). Approximately 300 μg of lysate protein were loaded in each of the test lanes, adjusted as follows to reflect equal amounts of β-galactosidase activity: untransfected H358 cells, 300 μg; pC53C1N + SV40 T, 180 μg; pC53C1N, 320 μg; pC53C1N + HPV-16 E6, 350 μg; C-33A, 150 μg.

support the hypothesis that the transcriptional activation function of p53 is closely linked with its ability to suppress cell proliferation. A human lung cancer cell line without endogenous p53 was used to study p53 transactivation in the present study and similar findings have recently been observed in yeast cells and in another human cell line which expresses low levels of endogenous p53 (Kern *et al.*, 1992; Scharer and Iggo, 1992).

The viral oncoproteins of several DNA tumor viruses namely SV40, adenovirus type 5 and the human papillomaviruses appear to function in cell transformation through specific interactions with cellular proteins that are involved in the control of cell growth (Lane and Crawford, 1979; Linzer and Levine, 1979; Sarnow *et al.*, 1982; Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Münger *et al.*, 1989b; Werness *et al.*, 1990). A number of studies demonstrate a correlation between p53 binding and transformation by these viruses (Whyte *et al.*, 1988; Peden *et al.*, 1989; Werness *et al.*, 1990; Zhu *et al.*, 1991). The results presented in this study indicate that viral oncoproteins such as SV40 T-ag and HPV-16 E6 can inhibit the function of p53 as a sequence-specific transcription factor and this ability correlates with the ability of the viral proteins to alter cell growth in immortalization/transformation assays.

SV40 T-ag and the E1B 55 kDa protein presumably inactivate p53 by sequestering it into oligomeric complexes which increase the stability of p53 but inhibit its ability to function (Oren *et al.*, 1981). Mutants of T-ag that have lost the ability to complex with p53 are at least partially defective for transformation (Peden *et al.*, 1989; Srinivasan *et al.*, 1989; Zhu *et al.*, 1991). For example, SV40 mutant 5080, which carries a single amino acid substitution (Pro584 → Leu), and mutants 2809 and 2811, which have small linker insertions (at amino acid positions 409 and 424 respectively), each encode mutant T-ags which fail to bind to p53 and are defective in transformation assays using primary rodent cells (Peden *et al.*, 1989; Zhu *et al.*, 1991). In the present study, cotransfection of wild-type T-ag reduced the ability of p53 to activate transcription, whereas coexpression of 5080, 2809 and 2811 did not affect p53 transactivation. Similarly, an SV40 deletion mutant that does not encode the p53-binding domain of T-ag was also unable to interfere with p53 transactivation in our assay.

The immortalizing activities of oncogenic HPVs reside in the gene products of two open reading frames, E6 and E7 (Hawley-Nelson *et al.*, 1989; Münger *et al.*, 1989a). The E7 protein of both high and low risk HPVs has been shown to bind to pRb although the high risk E7 proteins bind with significantly higher affinities (Dyson *et al.*, 1989; Münger *et al.*, 1989b). The E6 oncoproteins of high risk but not low risk HPVs form detectable complexes with p53 (Werness *et al.*, 1990). The association of E6 with p53 *in vitro* leads to a selective degradation of p53 via a ubiquitin-dependent proteolysis system (Scheffner *et al.*, 1990). We have now shown that coexpression of the high risk HPV type 16 E6 oncoprotein efficiently abrogates transcriptional transactivation by wild-type p53 but that low risk HPV-11 E6 or a mutant of HPV-16 E6 which is defective for p53 binding were unable to affect this function. The results of the present study provide strong evidence that the E6 protein from HPV-16 and presumably the E6 proteins encoded by other anogenital-specific oncogenic HPVs interact with p53 *in vivo*.

The mechanisms by which T-ag and HPV-16 E6 inhibit p53 transactivation are unclear. A recent study by Farmer *et al.* (1992) suggests that the observed interaction between T-ag and p53 may prevent p53 from binding specifically to target DNA sequences. With regard to E6, the low levels of transactivation may reflect the ability of E6 to degrade p53. However, p53's ability to function in transactivation may also be altered by the formation of stable p53−E6 complexes. Preliminary data suggest that coexpression of HPV-16 interferes with the DNA-binding function of p53 (T.Unger and J.Mietz, unpublished findings).

In a recent report by Yew and Berk (1992), the effects of the E1B 55 kDa protein on p53 transactivation were examined using GAL4−p53 fusion constructs. Wild-type E1B 55 kDa protein was found to inhibit GAL4−p53 transactivation and the ability of various E1B mutants to affect transactivation strongly correlated with their ability to transform primary rodent cells in cooperation with adenovirus E1A protein. p53 binding, however, did not strictly correlate with the ability to inhibit transactivation in the E1B study as one E1B 55 kDa mutant that was able to bind p53, but defective for transformation did not significantly inhibit p53 transactivation. In addition, the adenovirus type 12 E1B 54 kDa protein, which does not bind p53 detectably, was also able to inhibit p53-mediated transactivation. These results suggest a further complexity to the mechanism by which at least adenovirus E1B affects transactivation and transformation through p53.

In conclusion, this study shows that the inhibition of the p53 transactivation function by SV40 T-ag and HPV E6 correlates with both the p53-binding properties and the transforming potential of these viral oncoproteins and suggest that cellular events that interfere with the transactivation function of wild-type p53 (i.e. mutation of the p53 protein or binding by certain viral oncoproteins) may result in the loss of its ability to function as a tumor suppressor.

Materials and methods

Plasmid constructs

Reporter plasmids 3FSVCAT and 6FSVCAT were constructed by insertion of three (3F) or six (6F) copies of the consensus p53 binding sequence TGCCT (Kern *et al.*, 1991) upstream of the enhancer-deleted SV40 promoter in the CAT reporter plasmid pA10CATBS (Spalholz *et al.*, 1987). Double-stranded oligonucleotides prepared from the following sequences: 5′-GATCTCCTTGCCTGGACTTGCCTGGCCTTGCCTTTTG-3′ (3F) and 5′-GATCTCCTTGCCTGGACTTGCCTGGCCTTGCCTTCCT TGCCTGGACTTGCCTGGCCTGCCTTTG-3′ (6F) were used to insert the p53 target sequences between the *BglII* and *SalI* sites of pA10CATBS. Oligonucleotides 3F and 6F were inserted in the opposite orientation to generate plasmids 3RSVCAT and 6RSVCAT.

Wild-type human p53 expression plasmid pC53-C1N and p53 mutant constructs pC53-Cx33 (Val143 → Ala), pC53-Cx22AN (Arg175 → His), pC53-248 (Arg248 → Trp), pC53-Cx4.2N (Arg273 → His) and pC53Cx7AN (Asp281 → Gly) were obtained from A.Levine (Princeton University, Princeton, NJ) and B.Vogelstein (Johns Hopkins University, Baltimore, MD). All of the p53 expression plasmids listed above employed the CMV promoter/enhancer and the SV40 early region polyadenylation signal.

The HPV-16 E6 expression plasmid p1221 was constructed by replacement of the SV40 T-ag sequences (*HindIII−HpaI*) in RSV T-Ag (Phelps *et al.*, 1988) with a multicloning site cassette generating the vector p1214 into which the HPV-16 E6 ORF (nts 104−559) was inserted (provided by K.Münger). The HPV-16 E6 ORF was reconstructed by linker addition to provide a favorable start site for translation (at nt 104) and restriction sites at either end to facilitate subcloning. A *SalI−HindIII* fragment containing the HPV-11 E6 ORF (described in Werness *et al.*, 1990) was inserted into RSV expression vector p1214 to create p2021. The HPV-16 E6 mutant JH26 has amino acid substitutions at residues 7 (Arg → Ser), 8 (Pro → Ala) and 9 (Arg → Thr) (J.Huibregtse, unpublished data). This mutant HPV-16 E6 gene was also subcloned into p1214 as a *SalI−HindIII* fragment and designated p2022.

The SV40 expression plasmid pPVU-0 includes the *BamHI* (2533) to *PvuII* (270) early region fragment from the wild-type SV40 genome inserted between the *BamHI* and *PvuII* sites of pBR322 (Kalderon and Smith, 1984). The SV40 T-ag point mutant 5080 has a substitution at amino acid 584 (Pro → Leu) (Peden *et al.*, 1989) and deletion mutant pIB147 contains a translational stop codon at amino acid 148 which terminates the large T-ag after amino acid 147 (Sompayrac and Danna, 1988). Plasmid pCC2Pa includes the entire SV40 genome inserted into the *EcoRI* site of pUC18 (Zhu and Cole, 1989). T-ag mutants inA2809 and inA2811 were generated from pCC2Pa and contain linker insertions that insert two or three amino acids at position 424 (2811) and 409 (2809) respectively (Zhu and Cole, 1989).

Transfection and CAT assays

All transient transfection assays were performed using H358, a human lung cancer-derived cell line devoid of any p53 expression. This cell line has a homozygous deletion of p53 genomic sequences and produces no detectable p53 mRNA or protein (Takahashi *et al.*, 1989). H358 cells were maintained in RPMI (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone) and 100 μg penicillin/streptomycin (Gibco BRL). Cells were plated at a density of ~3−5 × 10^5 per 60 mm dish 18 h prior to transfection. Cells were transfected using the calcium phosphate coprecipitation procedure essentially as previously described by Phelps *et al.* (1988). Each 60 mm dish received 15 μg of DNA, with the total amount adjusted by the addition of salmon sperm DNA. Cotransfections included 4 μg of CAT reporter plasmid, the indicated amounts of p53 plasmids and where indicated, 8 μg of viral oncoprotein expression plasmids. A β-galactosidase expression plasmid, pCMVβ (Clonetech Laboratories, Palo Alto, CA) (1.5 μg) was included in each DNA mixture as a marker for transfection efficiency. Cell extracts were first assayed for β-galactosidase activity and CAT assays were carried out as described by Phelps *et al.* (1988) using aliquots containing a defined amount of β-galactosidase activity from each extract.

Immunoblotting

H358 cells were transfected using 50 μg of lipofectin according to the manufacturers recommendations (Gibco BRL) with 1 μg pC53C1N, 3 μg pCMVβ and, where indicated, 8 μg of either SV40 large T-ag or HPV-16 E6 expression plasmids. At 48 h post-transfection, the cells were washed twice with PBS, scraped into tubes and an aliquot of each sample was set aside for β-galactosidase determination. The remaining cells were pelleted and 100 μl lysates were prepared essentially as described by Unger *et al.* (1992) except that 1.0% rather than 0.1% Nonidet P-40 was used in the lysis buffer. Samples containing ~300 μg of protein extract were fractionated by SDS−PAGE followed by immunoblotting. Mouse monoclonal antibody pAb1801 (Oncogene Sciences Mineola, NY) was used to detect p53. A horseradish peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories) was used in conjunction with an enhanced chemiluminescence system (Amersham) for detection with pAb1801. Mouse monoclonal antibody pAb416 (Oncogene Sciences Mineola, NY) was used to detect SV40 T-ag. A ^{125}I-labeled sheep anti-mouse antibody (Amersham) was used for detection with pAb416.

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