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SHORT COMMUNICATION

Repression of p53 Transcriptional Activity by the HPV E7 Proteins

PAOLA MASSIMI and LAWRENCE BANKS[1]

International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34012 Trieste, Italy

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The major transforming protein of human papillomaviruses (HPVs) is encoded by the E7 gene. This protein cooperates with activated oncogenes to transform primary rodent cells and with the viral E6 gene to immortalize primary human keratinocytes. Numerous cellular targets of HPV E7 have now been identified including pRb, p107, cyclin A, TATA box binding protein (TBP), and members of the AP-1 transcription factor family. As with Adenovirus E1a, many of these interactions are important for the ability of E7 to transform cells. Recent studies have demonstrated that Adenovirus E1a can also inhibit the transcriptional activity of the cellular tumor suppressor protein, p53. We have performed a series of analyses to determine whether HPV E7 proteins share this characteristic. We show that HPV E7 proteins derived from both benign and tumor-associated HPV types are able to inhibit p53 transcriptional activity. Mutational analysis of the HPV-16 E7 protein reveals that a key domain involved in mediating this activity is the casein kinase II (CKII) recognition site, which has been shown to modulate E7 binding to TBP. We further show that E7 does not bind to p53 directly, but will do so in the presence of exogenously added TBP and that this binding is increased following CKII phosphorylation. These results suggest that the E7–TBP interaction may be responsible for inhibiting p53 transcriptional activity.

Human Papillomaviruses (HPVs) associated with tumor development encode three transforming proteins, E5, E6, and E7, of which E7 is the most potent (*1–5*). This protein cooperates with activated oncogenes in the transformation of primary rodent cells and with E6 in the immortalization of primary human keratinocytes (*6, 7*). Both E6 and E7 continue to be expressed in cells derived from cervical tumors (*8–10*) and the continuous expression of both proteins is required for maintenance of the transformed phenotype in rodent cells (*11, 12*). In common with Adenovirus E1a protein, HPV-16 E7 binds to a number of cellular proteins which are intimately involved in regulating cell growth. These include pRb, p107, Cyclin A, TBP, and the AP-1 family of transcription factors (*13–17*). HPV-16 E7 has been shown to be capable of immortalizing human cells at low frequency in the absence of other cooperating oncogenes (*18, 19*). This can be explained by the fact that E7 can overcome p53-induced growth arrest which is mediated by p21, through the premature release of E2F from pRb, and thus E7 shortcuts the inhibitory function of the p21 protein (*20–22*). A similar strategy is also used by Adenovirus E1a, therefore both viral proteins have evolved a mechanism for overcoming a p53 regulatory pathway indirectly. Recent studies with Adenovirus E1a demonstrated an additional activity of this protein with respect to p53 function: the ability to inhibit p53's transcriptional activity (*23*). These studies suggested that this may be brought about through an increase in the molecular weight of p53 protein complexes within the cell, in response to the presence of E1a. We were, therefore, particularly interested in determining whether the HPV E7 proteins could also inhibit p53 transcriptional activity.

To investigate this we first performed a series of experiments to determine whether HPV-16 E7 could modulate p53 transcriptional activity in p53-null Saos-2 cells. Cells (1×10^5) were transfected with 1 μg of the p53 responsive CAT reporter plasmid, pG13CAT, plus 1 μg of p53 expression plasmid. Increasing amounts of the HPV-16 E7 expression plasmid, pJ4Ω.16E7, were included and the cells were harvested after 48 hr. CAT assays were performed as described previously (*24*) and the results obtained are shown in Fig. 1A. It is clear that increasing amounts of HPV-16 E7 produce a marked decrease in p53 transcriptional activity in a manner similar to that reported for Adenovirus E1a protein (*23*). This result indicates that HPV-16 E7 can also inhibit p53 transcriptional activity.

We were then interested to determine whether this function of E7 was restricted to the tumor-associated E7 proteins or was conserved throughout the genital HPV types. To do this, the above assay was repeated but included HPV-18 E7, HPV-6 E7, and HPV-11 E7 (*5*). The results shown in Fig. 1B demonstrate that all four E7 proteins possess the ability to inhibit p53 transcriptional

[1] To whom correspondence and reprint requests should be addressed.

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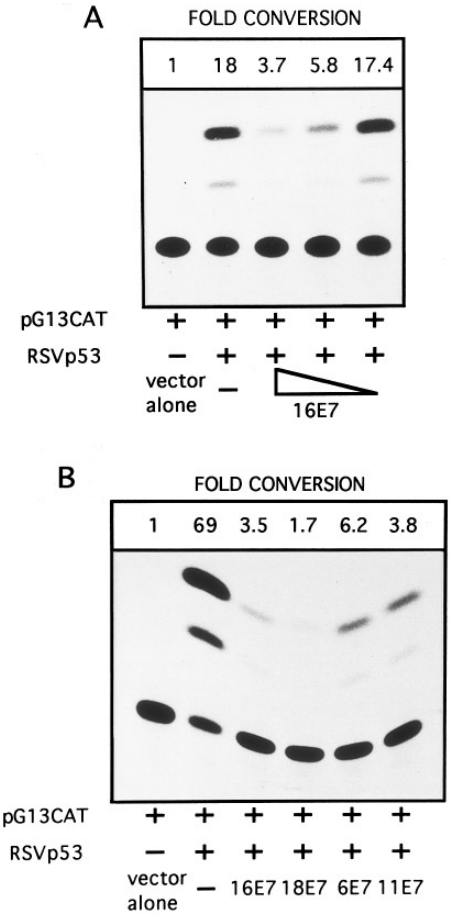

FIG. 1. Abolition of p53-mediated transcriptional activation by HPV E7 proteins. Saos-2 cells were transfected with 1 μg of the p53 responsive CAT plasmid, pG13CAT, 1 μg of the RSVp53 plasmid together with pJ4Ω or pJ4Ω.E7 plasmids as indicated. Cells were harvested and CAT activity was measured after 48 hr. (A) Titration of pJ4Ω.16E7 as indicated containing 10, 5, and 1 μg of the transfected E7 expression plasmid. (B) Comparison of different E7 proteins derived from benign and tumor-associated HPVs. In each case 10 μg of the E7 expression plasmid was transfected.

activity. These results suggest that this function of E7 is conserved between both benign and tumor-associated HPV types and is not directly associated with the ability of E7 to transform cells.

Having shown that HPV E7 proteins will inhibit p53 transcriptional activity we were then interested in identifying the region of the E7 protein which was responsible. Therefore a series of previously well-characterized HPV-16 E7 mutants (*25–27*) were assessed for their ability to inhibit p53 transcriptional activity. The results obtained are shown in Fig. 2A. Mutants of E7 which are defective in transformation, such as 566 (*26*) and 631 (*25*), still retain the ability to inhibit p53 transcriptional activity. It is of particular note that the 631 mutant is defective in pRb binding, and this demonstrates therefore that inhibition of p53 transcriptional activity is independent of E7's ability to bind pRb. These results also indicate that this activity of E7 does not reflect its ability to transform cells. In fact, only two of the mutants tested, 638 (*25*) and 31/32 (*27*), appeared to have a reduced ability to inhibit p53 transcriptional activity. Mutant 638 lies within the pRb binding pocket, but has been shown previously to encode an unstable protein (*25*). Mutant 31/32, on the other hand, encodes a stable E7 protein which is defective for CKII recognition (*27*). This result indicates that CKII phosphorylation of E7 may play a role in its inhibition of p53 transcriptional activation. Previous studies have also shown E7 to be potentially phosphorylated at other residues within the protein (*28*). We were therefore interested to see if phosphorylation at these sites might be important for the ability to suppress p53 transcriptional activity. Four additional mutants of E7 were assayed and the results obtained are shown in Fig. 2B. It is clear from this analysis that only phosphorylation of the CKII site, as shown by the p32 and p31/32 mutants, appears to be

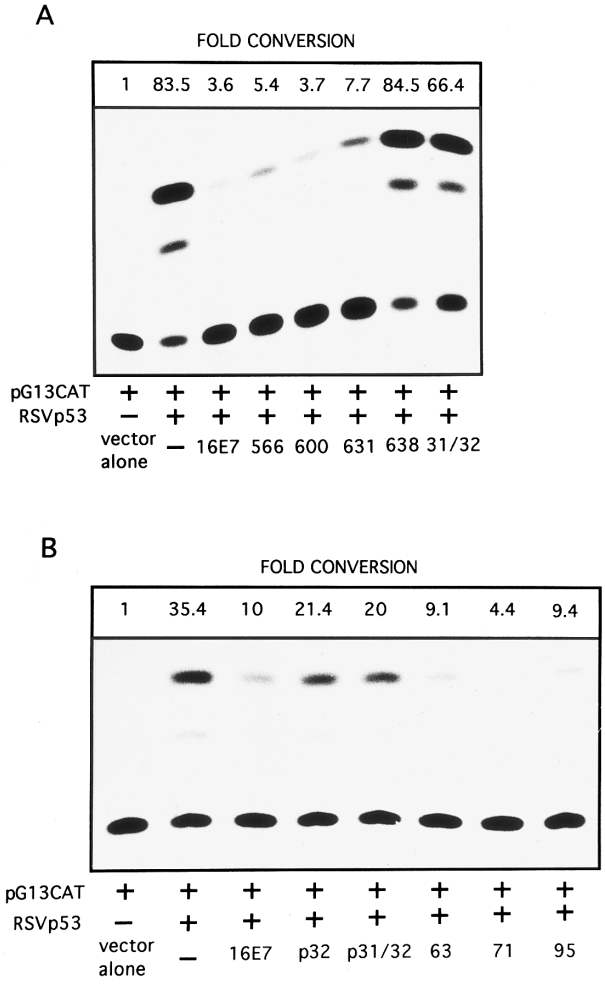

FIG. 2. Localization of the region of HPV-16 E7 responsible for inhibition of p53-mediated transcriptional activation. Saos-2 cells were transfected and processed as described in the legend of Fig. 1. (A) Mutants of E7 spanning the conserved amino terminal region of the protein. (B) Phosphorylation-defective E7 mutants which are spread throughout the protein.

TABLE 1

HPV E7-Mediated Repression of p53 Transcriptional Activation

Expression plasmid	Fold reduction[a]
pJ4Ω	1.0
pJ4Ω.16E7	5.3
pJ4Ω.18E7	4.7
pJ4Ω.6E7	4.9
pJ4Ω.11E7	2.9
pJ4Ω.566	4.8
pJ4Ω.631	3.1
pJ4Ω.638	1.0
pJ4Ω.p32	1.8
pJ4Ω.p31/32	1.6
pJ4Ω.p63	4.9
pJ4Ω.p71	9.5
pJ4Ω.p95	5.5

Note. The mutants of E7 are as follows: 566 represents amino acid residue(aa) 2 His-Pro; 631 represents aa24-Cys-Pro; 638 represents aa26 Glu-Gly; p32 represents aa32-Ser-Trp; p31/32 represents aa31 Ser-Arg and aa32 Ser-Pro; p63 represents aa63 Ser-Ala; p71 represents aa71 Ser-Gly; p95 represents aa95 Ser-Ala.

[a] Numbers show fold reduction in p53 transcriptional activation obtained by cotransfecting 10 μg of the indicated expression plasmids together with 1 μg of pG13CAT and 1 μg of p53 expression plasmid. These represent the mean values from between 3 and 6 individual experiments.

important for E7's inhibition of p53 transcriptional activity. Table 1 shows a summary of the above assays from a series of at least three independent transfections.

Although the above studies show that E7 will inhibit p53 transcriptional activity on a CAT reporter construct, a major concern is whether or not E7 can affect p53 induction of a naturally occurring target gene. One such gene encodes the p21/WAF-1 protein (*20, 29*). Therefore, we proceeded to investigate the effects of E7 expression upon p21 induction in Saos-2 cells following transfection with a p53 expression plasmid. Cells were transfected as described above and, following extraction as described previously (*30*), the p21 and p53 protein levels were determined by Western blot analysis. The results obtained are shown in Fig. 3. Figure 3A shows the level of p53 protein obtained following transfection of Saos-2 cells with 1 μg of RSVp53 plus 10 μg of pJ4Ω.16E7, as indicated. As can be seen, a slight increase in the level of p53 protein is obtained in cells containing E7 and this agrees with previous studies (*31*). In contrast, when the same Western blot was reprobed for p21, it is quite clear that there is a decrease in the level of p21 protein in the E7 containing cells, compared with the cells transfected with p53 alone. These results demonstrate that, in a transient transfection, E7 can significantly reduce the level of expression of a naturally occurring p53-induced protein.

We have recently shown that CKII phosphorylation of E7 stimulates its ability to complex with the core component of TFIID, the TATA box binding protein (TBP) (*16*). Since both E1a and p53 associate with TBP, a possible explanation of the higher molecular weight forms of p53 seen in E1a expressing cells (*23*) could be coassociation of E1a-TBP and p53, which might indicate a mechanism for inhibition of p53 transcriptional activity. To test this hypothesis we performed a series of *in vitro* binding assays to determine whether E7 and p53 could associate in the presence of TBP. HPV-16 E7 and p53 were expressed as GST fusion proteins and purified on glutathione-agarose columns. They were then incubated with the appropriate *in vitro*-translated proteins; results of the binding assays are shown in Fig. 4. As can be seen from Fig. 4A, very little p53 is bound to the GST.16E7 protein when compared with the GST control. However, upon addition of *in vitro*-translated TBP there is a dramatic increase in the amount of p53 retained on the column. The converse experiment is shown in Fig. 4B. In the absence of additional TBP, no E7 is bound to the GST.p53 column; upon the addition of TBP, a stimulation of E7 binding is seen. These results demonstrate that E7 and p53 will normally not associate directly. However, in the presence of TBP a tripartite complex can form, thus indicating that both E7 and p53 can bind TBP simultaneously.

Having shown previously that CKII phosphorylation of E7 increases TBP binding (*16*) we then repeated the above binding experiment with a mutant E7 GST.p31/32 fusion protein, both before and after CKII phosphorylation of the fusion proteins. Phosphorylation reactions were done as described previously (*16*), and the results of the triple binding assay are shown in Fig. 4C. It is clear that CKII phosphorylation of the wild-type GST–E7 fusion protein increases the level of TBP binding and this agrees with our previous observations (*16*). In addition, this stimulation of TBP binding is accompanied by a dramatic increase in the amount of p53 protein retained, further supporting the notion that the binding of p53 to E7 is via TBP. In contrast, performing the same experiment with the mutant GST.p31/32 protein shows that,

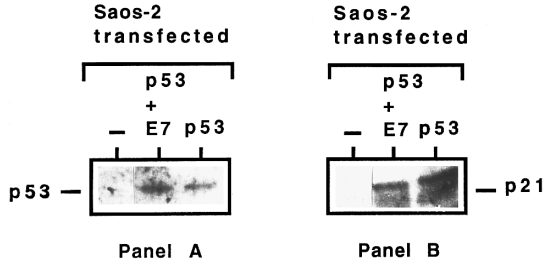

FIG. 3. Inhibition of p21 protein induction by pJ4Ω.16E7. Saos-2 cells were transfected with either 1 μg RSVp53 (p53) plasmid plus 10 μg pJ4Ω.16E7 (E7) plasmid, as indicated, or vector alone sequences (−). After 48 hr the cells were extracted and the p53 protein levels (A) measured using a pool of anti-p53 monoclonal antibodies pAb1801, 1802, and 1803 (*32*). p21 protein levels (B) were measured using an anti-p21 antibody (Oncogene Science Inc.). Western blots were developed using the Amersham ECL detection kit according to the manufacturer's instructions.

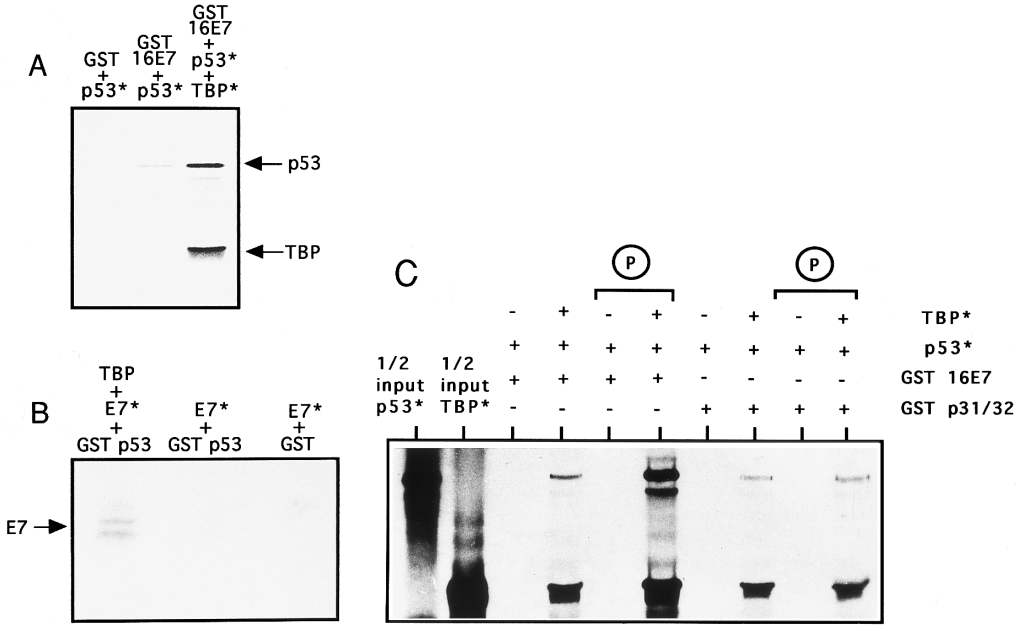

FIG. 4. Complex formation between p53, TBP, and E7. (A) *In vitro*-translated labeled p53 (p53*) was added to GST resin or GST-16E7 resin and binding monitored. In the last lane radiolabeled TBP (TBP*) was also included. The location of the p53 and TBP proteins are shown. (B) Binding of labeled *in vitro*-translated HPV-16 E7 (E7*) to GST resin and GST-p53 resin. In the first lane cold TBP was also added to the reaction. The presence of E7 protein is indicated. (C) Binding of *in vitro*-translated labeled p53 (p53*) and *in vitro*-translated labeled TBP (TBP*) to GST.16E7 resin and mutant GST.p31/32 resin before and after CKII phosphorylation (P). Binding reactions were performed in PBS as described previously (*16*) and p53 and TBP protein inputs are shown. Quantitation of the bound proteins with a PhosphorImager indicates that the percentage of TBP retained rises from 6 to 19.5% following phosphorylation of wild-type E7, and bound p53 goes from 6 to 19% at the same time. The level of proteins retained on the p31/32 resin does not change following phosphorylation and remains at 4.8% for TBP and 5% for p53.

although p53 protein is bound with TBP in the absence of phosphorylation, the level of neither protein changes following the CKII phosphorylation reaction. It is noteworthy that the 31/32 mutant of E7 retains the ability to bind TBP, and hence p53, in a manner like wild-type E7 in the absence of phosphorylation. This is not surprising considering the results of the *in vivo* assays where this mutant also weakly inhibits p53 transcriptional activity. However, phosphorylation of the wild-type E7 protein greatly increases its ability to bind TBP and this correlates very closely with its increased ability to inhibit p53 transcriptional activity *in vivo.*

In this series of experiments we have demonstrated that the HPV E7 proteins will inhibit p53 transcriptional activity in a manner similar to that observed for Adenovirus E1a. This activity is conserved between proteins derived from both benign and tumor-associated HPVs and hence most probably represents a function of E7 related more to viral replication than to transformation. Indeed, mutational analysis of the HPV-16 E7 protein demonstrates that mutants defective in transformation and pRb binding nonetheless retain the ability to inhibit p53 transcription. In contrast, a mutant of E7 defective in its CKII recognition site had a markedly reduced ability to inhibit p53 transcriptional activity. Based on our previous demonstration that CKII phosphorylation stimulates E7 complex formation with TBP, it is tempting to speculate that it is the association with TBP which mediates this inhibition of p53 transcription. This hypothesis is supported by the results from the *in vitro* binding assays in which we show that E7 and p53 will associate in the presence of exogenously added TBP and that this binding increases following CKII phosphorylation of the E7 protein. These results suggest an additional route by which HPV can perturb normal p53 function.

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