Comparison of human papillomavirus type 18 (HPV-18) E6-mediated degradation of p53 in vitro and in vivo reveals significant differences based on p53 structure and cell type but little difference with respect to mutants of HPV-18 E6

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An important characteristic of the E6 proteins derived from oncogenic associated human papillomaviruses (HPVs) is their ability to target the cellular tumour suppressor protein, p53, for ubiquitin mediated degradation. Several studies have attempted to address the important characteristics of both E6 and p53 for this activity in vitro, but the equivalent determinants have not been extensively assessed in vivo. Indeed, recent studies indicate differences between the in vitro and the in vivo degradation assays. We have performed an extensive analysis of the ability of a range of HPV-18 E6 mutants to direct p53 degradation in vivo. In addition, we have also compared the ability of HPV-18 E6 to direct the degradation of different oligomeric forms of p53 both in human and in murine cells. The results of these studies show that mutants of E6 exhibit very similar phenotypes both in vitro and in vivo. In contrast, mutants of p53 show markedly different susceptibilities in vitro and in vivo to E6-induced degradation, and this is further affected by the nature of the cell type in which the assays are performed. Finally, using a cell line temperature sensitive for the E1 ubiquitin-activating enzyme we have been able to show directly that this enzyme is involved in the process of E6-mediated degradation of p53 in vivo.

Introduction

The p53 gene and its protein product have become the centre of intensive study since it became clear that slightly more than 50% of human cancers contain mutations in this gene (Hollstein et al., 1994). p53 is involved in the control of cell proliferation and in the arrest of cell cycle progression after DNA damage; under appropriate conditions it also participates in the mechanism of apoptosis (Diller et al., 1990; Lin et al., 1992; Lane, 1992; Kessis et al., 1993; Waga et al., 1994). p53 is a nuclear DNA-binding protein which acts as a transcriptional regulator of genes that possess a p53 responsive element in their promoter (Kern et al., 1991; Funk et al., 1992). Several cellular genes that are regulated by p53 such as waf-1/p21 (El-Deiry et al., 1993), cyclin G (Okamoto & Beach, 1994) and gadd45 (Kastan et al., 1992) have been demonstrated to participate in the regulation of cell cycle progression and, as such, are good candidates for downstream mediators of p53 function.

To avoid the suppression of cell growth mediated by p53, some DNA tumour viruses have developed specific strategies to abrogate its function. High risk type human papillomaviruses (HPVs), such as HPV-16 and HPV-18, which are associated with the development of cervical cancer, encode an E6 oncoprotein which binds to p53 and stimulates its rapid degradation via the ubiquitin proteolytic pathway (Scheffner et al., 1990). A 100 kDa cellular protein, E6-AP, is involved in the E6–p53 interaction and, in conjunction with E6, acts as a ubiquitin–protein ligase (Huibregtse et al., 1991, 1993; Rolfe et al., 1995). It has also been shown that E6 can abolish p53 transcriptional activation (Lechner et al., 1992; Pim et al., 1994; Thomas et al., 1995) and immortalize primary cells (Band et al., 1993; Pim et al., 1994) independently of its ability to promote p53 degradation in vitro. Thus the role of E6-mediated degradation of p53 during HPV oncogenesis is still unclear.

Extensive mutational analysis has demonstrated a high degree of conservation of function between the E6 proteins of HPV-16 and HPV-18 and has allowed the identification of critical domains of the E6 molecule necessary both for the association with p53 and for the subsequent stimulation of p53 degradation in vitro (Crook et al., 1991; Mietz et al., 1992, Pim
Some studies have also been done on the ability of different HPV-16 E6 mutants to direct p53 degradation in vivo, and although there is a good consensus between the in vivo and in vitro assays, there are nonetheless mutants of E6 which behave differently between the two assays (Foster et al., 1994; Dalal et al., 1996). In addition, although several studies have analysed the ability of E6 to label different oligomeric forms of p53 for degradation in vitro (Thomas et al., 1995; Marston et al., 1995; Li & Coffino, 1996) there is little information on this activity in vivo. Both of these aspects are particularly relevant in the light of recent studies which have shown that certain mutants of p53 were not susceptible to E6-mediated degradation in vitro, but were susceptible in vivo (Crook et al., 1996). In order to clarify this situation we have established an in vivo assay for following E6-mediated degradation of p53 in p53 null murine and human cells. Using this system we have analysed the ability of the different HPV-18 E6 mutants to direct p53 degradation in vivo and also analysed the susceptibility of a set of oligomerization defective p53 mutants to E6-mediated degradation in vivo. We have also extended this analysis to investigate the role of the E1 ubiquitin-activating enzyme in E6-induced degradation of p53 in vivo. The results of these studies show that the different E6 mutants largely display very similar phenotypes with respect to p53 degradation in vitro and in vivo. In contrast, significant differences are obtained with the different oligomerization defective mutants of p53, depending both upon whether the assays are performed in vitro or in vivo, and also on whether the assays are done in a human or murine cell background. Finally, using a cell line expressing a temperature sensitive E1 ubiquitin-activating enzyme, we have been able to demonstrate that this enzyme contributes directly to the process of E6-mediated degradation of p53 in vivo.

**Methods**

- **Plasmids.** The p40 expression plasmids containing the wild-type and HPV-18 E6 mutants have been described previously (Pim et al., 1994). Wild-type, 518, 338 and 1262 mutants of p53 were expressed under the control of the CMV promoter and have also been described previously (Tarunina & Jenkins, 1993).

- **Cells and tissue culture.** Human Saos-2 osteosarcoma and p53 null 10(1) cells were grown in DMEM with 10% foetal calf serum. The E1 ts mouse cells were grown under the conditions described previously (Chowdary et al., 1994).

  All the transient transfections were carried out using the calcium phosphate precipitation procedure (Matlashewski et al., 1987) using plasmids and conditions as indicated in the text. Parallel transfections with the lacZ expression plasmid pCH110 were also performed to monitor transfection efficiencies which were routinely of approximately 10%. No significant differences in transfection efficiencies were noted between the murine and human cells.

- **In vivo degradation assays.** Cells were harvested in extraction buffer (250 mM NaCl, 0-1% NP40, 50 mM HEPES pH 7.0 and 1% Aprotinin) 24–48 h after transfection and equal amounts of protein were run on a polyacrylamide gel and transferred to nitrocellulose. The levels of p53 protein were determined by probing the Western blots with a mixture of the anti-human p53 specific monoclonal antibodies Pab1801 and 1803 (Banks et al., 1986), followed by anti-mouse biotin conjugate antibody (Dako) and avidin-peroxidase conjugate (Dako). The blots were developed using the Amersham ECL technique according to the manufacturer’s instructions. Western blots were reprobed with an anti-ADP-ribosyltransferase (ADP–RT) antibody to verify equal levels of protein loading. For quantification of the level of p53 degradation, the autoradiographs were densitometrically scanned using a Bio-Rad Imaging Densitometer.

**Results**

**Identification of domains of HPV-18 E6 essential for mediating p53 degradation in vivo**

The HPV-18 E6 protein contains four Cys-X-X-Cys motifs which may mediate zinc binding and the formation of two zinc-finger structures (Matlashewski et al., 1986; Barbosa et al., 1989). A previous mutational analysis of the HPV-18 E6 protein identified a number of domains on the protein important for its ability to target p53 for degradation in vitro (Pim et al., 1994). These were found to consist of three broad regions of the protein as defined by mutants ΔF(1113–117) and ΔG(1126–130) within the carboxy-terminal loop; ΔA–(A47–49), ΔI(Δ66–39) and ΔI(Δ55–56) within the amino-terminal loop; and finally a stretch of amino acids prior to the amino-terminal loop as defined by mutants ΔD(Δ21–25), M2(105/11G) and AN terminus(Δ4–7). Many of these domains have also been identified as essential for these functions in HPV-16 E6 and this demonstrates a high degree of conservation of function between the E6 proteins of HPV-16 and -18 (Crook et al., 1991; Pim et al., 1994).

Considering the recent studies which showed variation between in vitro and in vivo degradation assays (Crook et al., 1996) we were interested in analysing the ability of this group of HPV-18 E6 mutants to target p53 for degradation in vivo. The assay we chose for this analysis consists of transfecting p53 null Saos-2 cells with a p53 expression plasmid, together with increasing amounts of an HPV-18 E6 expression plasmid.

![Fig. 1. HPV-18 E6-mediated degradation of p53 in vivo. Human p53 null Saos-2 cells were co-transfected with 5 µg of p53 expression plasmid and the indicated amounts (µg) of HPV-18 E6 expression plasmid. After 48 h the cell proteins were extracted and equal amounts were separated by SDS–PAGE. The p53 protein levels were ascertained by probing the Western blots with a mixture of the anti-human p53 specific monoclonal antibodies Pab1801 and Pab1803 (Banks et al., 1986).](image-url)
After 48 h the levels of p53 protein expression can be determined by Western blot analysis using a panel of anti-p53 monoclonal antibodies (Banks et al., 1986). The result of one such assay is shown in Fig. 1. As can be seen, increasing the amount of HPV-18 E6 produces a significant decrease in the levels of p53 expression, consistent with previous observations (Hauberk et al., 1992; Foster et al., 1994). At the 5 µg input of HPV-18 E6 there is complete degradation of the p53 protein, and these conditions were chosen for the following experiments. We then proceeded to investigate the ability of the different HPV-18 E6 mutant proteins to target p53 for degradation in vivo; the results from a representative assay are shown in Fig. 2. It is clear that some of the mutants, such as ΔA and ΔD, are defective in their ability to target p53 for degradation in vivo. Other mutants, such as ΔG, M2 and ΔF, exhibit a low activity when compared with the wild-type protein. Mutants exhibiting an intermediate phenotype are represented by ΔK and ΔN terminus. The results from a series of in vitro and in vivo p53 degradation assays are summarized in Table 1. In general, there is a very good correlation between the two sets of assays and the mutant E6 proteins which failed to degrade p53 in vitro were also defective in vivo. Only one mutant, ΔN terminus, appeared significantly different between the two assays. In vivo, this mutant is largely defective in its ability to target p53 for degradation, but in vitro a significant level of activity was consistently obtained. These results suggest that at least for HPV-18 E6, the ability to direct p53 degradation in vitro correlates closely with its ability to direct

Table 1. Comparison of the ability of different HPV-18 E6 mutant proteins to direct p53 degradation in vitro and in vivo

<table>
<thead>
<tr>
<th>E6 protein</th>
<th>p53 degradation in vitro</th>
<th>p53 degradation in vivo</th>
<th>% of p53 degradation in vivo†</th>
</tr>
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<tbody>
<tr>
<td>wt</td>
<td>+ + +</td>
<td>+ + +</td>
<td>80 ± 5 ± 8 ± 0</td>
</tr>
<tr>
<td>ΔA (Δ47–49)</td>
<td>+/−</td>
<td>+/−</td>
<td>60 ± 1 ± 2</td>
</tr>
<tr>
<td>ΔB (Δ94–97)</td>
<td>+/−</td>
<td>+/−</td>
<td>30 ± 0 ± 5 ± 0</td>
</tr>
<tr>
<td>ΔD (Δ21–25)</td>
<td>+/−</td>
<td>+/−</td>
<td>12 ± 5 ± 4 ± 5</td>
</tr>
<tr>
<td>ΔI (Δ36–39)</td>
<td>+/−</td>
<td>+/−</td>
<td>70 ± 0 ± 11 ± 0</td>
</tr>
<tr>
<td>ΔK (Δ79–81)</td>
<td>+</td>
<td>+/−</td>
<td>18 ± 5 ± 1 ± 1</td>
</tr>
<tr>
<td>ΔL (Δ86–91)</td>
<td>+/−</td>
<td>+/−</td>
<td>41 ± 0 ± 9 ± 0</td>
</tr>
<tr>
<td>M2 (105/111G)</td>
<td>+/−</td>
<td>+/−</td>
<td>68 ± 0 ± 4 ± 3</td>
</tr>
<tr>
<td>N-TA (Δ4–7)</td>
<td>+/−</td>
<td>+/−</td>
<td>42 ± 5 ± 5 ± 5</td>
</tr>
<tr>
<td>ΔF (Δ113–117)</td>
<td>+</td>
<td>+/−</td>
<td>40 ± 0 ± 9 ± 0</td>
</tr>
<tr>
<td>ΔG (Δ126–130)</td>
<td>+/−</td>
<td>+/−</td>
<td>85 ± 0 ± 5 ± 0</td>
</tr>
<tr>
<td>ΔH (Δ144–149)</td>
<td>+</td>
<td>+/−</td>
<td>68 ± 0 ± 3 ± 4</td>
</tr>
<tr>
<td>M5 (114G)</td>
<td>+</td>
<td>+/−</td>
<td>68 ± 0 ± 3 ± 4</td>
</tr>
</tbody>
</table>

* From Pim et al. (1994).
† The percentage of p53 degradation was determined by densitometric scanning of the Western blot and the value represents the reduction in amount of p53 compared with the level of p53 in the absence of E6. The numbers represent the mean values from at least three independent experiments and the standard deviations are shown. The value for M5 is the mean ± 2 independent experiments.
p53 degradation in vivo, although subtle differences between the two assay systems clearly exist, as highlighted by the ΔN terminus mutant.

Susceptibility of oligomerization defective mutants of p53 to E6-mediated degradation is cell type dependent

Previous studies have shown that certain p53 oligomerization defective mutants were resistant to E6-mediated degradation in vitro whereas others appeared to be as susceptible as the wild-type p53 protein (Thomas et al., 1995; Marston et al., 1995). We had previously demonstrated that the dimeric form of p53, represented by mutant 518, was as susceptible as wild-type protein to E6-induced degradation whereas both of the monomeric mutants, 338 and 1262, were equally very weakly degraded in vitro (Thomas et al., 1995). Other studies had suggested that oligomerization did not play a role in the susceptibility of p53 to E6-induced degradation but, rather, this susceptibility was more influenced by the conformation of the p53 protein (Marston et al., 1995). To investigate these aspects further we proceeded to analyse the susceptibility of the previously defined p53 oligomerization defective mutants (Tarunina & Jenkins, 1993) to HPV-18 E6-induced degradation in vivo. The assays were performed as described above and were done in both human Saos-2 cells and murine 10(1) cells. The results obtained are shown in Fig. 3 (Saos-2) and Fig. 4 (murine 10(1)). In human cells the wild-type, the dimeric (518) and the monomeric (1262) forms of p53 are degraded by E6 with similar levels of efficiency, whereas the monomeric 338 mutant is degraded to a lesser extent. In murine cells, although the total degradation is less than that seen in Saos-2 cells, the wild-type and the dimeric forms of p53 are degraded with equal efficiency, the monomeric form 1262 is somewhat more resistant to E6-mediated degradation and the 338 mutant is completely resistant to E6-mediated degradation. A comparison of these results with those previously obtained in vitro is shown in Table 2 and

<table>
<thead>
<tr>
<th>p53</th>
<th>In vitro degradation*</th>
<th>Human cells</th>
<th>Mouse cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Saos-2</td>
<td>p53-null 10(1)</td>
</tr>
<tr>
<td>wt</td>
<td>++</td>
<td>88±0.4±2.0</td>
<td>70±0.9±6.0</td>
</tr>
<tr>
<td>518 (dimeric)</td>
<td>++</td>
<td>86±0.4±4.7</td>
<td>60.5±6.5</td>
</tr>
<tr>
<td>338 (monomeric)</td>
<td>+</td>
<td>52±0.4±2.6</td>
<td>0</td>
</tr>
<tr>
<td>1262 (monomeric)</td>
<td>+</td>
<td>80±0.4±4.0</td>
<td>35±0±4.0</td>
</tr>
</tbody>
</table>

* From Thomas et al. (1995).  
† The percentage of p53 degradation was determined as described for Table 1. Numbers represent the mean of at least three independent experiments and the standard deviations are shown. No change in the 338 protein levels was obtained at any time in the p53 null 10(1) cells.
demonstrates some interesting differences between the two assays. This is most clearly demonstrated by the two monomeric mutants. In human cells it is clear that the 1262 monomeric mutant is degraded with almost wild-type efficiency, in contrast to the results obtained in vitro. The 338 mutant is also susceptible to E6-induced degradation in human cells, again in contrast to the results from the in vitro assays. In murine cells, however, 1262 is much less susceptible to E6-induced degradation and mutant 338 is completely resistant. These results demonstrate the potential for significant differences in the in vitro and the in vivo degradation assays and that these can be further compounded by whether the assays are performed in human or murine cells.

The E1 ubiquitin-activating enzyme is essential for E6-mediated degradation of p53 in vivo

The proteolytic ubiquitin pathway involves the participation of several enzymatic activities. In a series of in vitro reconstitution studies using purified components, E6-mediated p53-ubiquitination was obtained and this demonstrated the requirement for three components of the ubiquitin pathway; the E1 ubiquitin-activating enzyme, the specific E2 conjugating enzyme UBC4 and the E3 ligase, which is the complex between HPV-18 E6 and the E6-AP protein (Rolfe et al., 1995). Antisense microinjection studies in HeLa cells also indicated the potential role of these components in the E6-mediated degradation of p53 (Rolfe et al., 1995). We were interested in investigating more directly the role of the E1 enzyme in vivo by using a cell line which contained a temperature sensitive (ts) E1 enzyme. This cell line had been used previously in studies which indicated that p53 may normally be regulated by the ubiquitination pathway in the absence of E6 protein (Chowdary et al., 1994). The ts cells were transfected with plasmids encoding p53 and HPV-E6 and were incubated at the permissive temperature for 16 h. Cells were then either shifted to 39 °C or kept at 33 °C for a further 24 h. The protein extracts were then prepared and processed as indicated in Fig. 1. (b) As (a) except the cells used were murine 10(1). An extract of C33-I cells was included as a positive control for p53 protein expression.

Discussion

In this study we have analysed the ability of different HPV-18 E6 mutant proteins to target p53 for ubiquitin mediated degradation in vivo and have also assessed the influence both of cell type and of p53 oligomerization upon this activity. Comparing the in vivo data with the in vitro data obtained previously (Pim et al., 1994; Thomas et al., 1995) it is clear that little difference exists with respect to E6. Thus, most of the mutants displayed similar phenotypes in vitro and in vivo. In contrast, significant differences were observed in the susceptibilities of p53 mutants depending upon whether the assays were performed in vitro or in vivo. These susceptibilities were...
also affected by whether the assays were performed in human or murine cells. These results suggest that the cellular environment may have a significant outcome on whether proteins are recognized and labelled for degradation by E6, but that this has little effect on the E6 protein itself.

Previous studies had identified at least three regions within the E6 proteins of both HPV-16 and HPV-18 which were essential for E6-mediated degradation of p53 in vitro (Crook et al., 1991; Pim et al., 1994). These fell within the extreme amino terminus of the protein, the amino-terminal loop and the carboxy-terminal loop. There have also been conflicting reports on the importance of E6-mediated degradation of p53 for transforming activity of E6. This was complicated both by observations in certain studies which suggested increased p53 turnover in cells immortalized by E6 proteins which were unable to direct p53 degradation in vitro (Band et al., 1993) and by lack of knowledge about the ability of E6 mutant proteins to target p53 degradation in vivo as opposed to in vitro (Pim et al., 1994). In the studies reported here, we have shown that very little difference exists between the results of in vitro and the in vivo degradation assays with the different HPV-18 E6 mutant proteins. This is largely in agreement with studies on a number of HPV-16 E6 mutants which also found few differences between in vitro and in vivo activity with respect to p53 degradation (Foster et al., 1994). This supports the argument that, at least in rodent cells, degradation of p53 is not required for HPV-18 E6 transforming activity, since it has been shown that E6 mutants deficient in in vitro p53 degradation were active in the ability to transform primary rodent cells (Pim et al., 1994). This latter point together with previous data showing that all of the E6 mutants are capable of transactivating the adenovirus E2 promoter (Pim et al., 1994) also suggests that the mutant E6 proteins are expressed at a functional level.

Only one mutant of HPV-18 E6, ∆N terminus, demonstrated activity in vivo when previously no activity had been observed in vitro. These results suggest that the cellular environment does not significantly alter E6 structure with respect to E6-mediated degradation of p53 and that the in vitro assays can be used as a reliable guide to E6 function in vivo. Several studies have been performed to investigate the susceptibility of p53 mutants to E6-mediated degradation in vitro (Thomas et al., 1995; Marston et al., 1995; Li & Coffino, 1996) although there have been few analyses in vivo. Indeed, a recent study showed that certain mutants of p53 which could not be degraded by E6 in vitro were nonetheless susceptible to E6-mediated degradation in vivo (Crook et al., 1996). This prompted our analysis of the ability of HPV-18 E6 to target the p53 oligomerization mutants for degradation in vivo. The dimeric mutant 518 appears to be equally well degraded both in vitro and in vivo, and displays a phenotype identical to that of the wild-type protein. This susceptibility to degradation does not appear to be greatly affected by whether the assays are performed in human or murine cells, although the efficiency is somewhat lower in murine cells. The situation is, however, more complex with the monomeric mutants of p53. In Saos-2 cells the monomeric mutant 1262 is degraded with an efficiency close to that of wild-type p53: this contrasts with the in vitro studies where this mutant was degraded more weakly than wild-type p53 (Thomas et al., 1995; Marston et al., 1995). A similar result, although not so striking, was obtained with the 338 monomeric mutant. This truncated form of p53 could also be degraded by HPV-18 E6 in Saos-2 cells, albeit not as efficiently as the wild-type protein. However, when the assays were performed in murine cells, results similar to those observed in vitro were obtained: 338 was completely resistant to E6-mediated degradation and reduced activity was seen with the 1262 mutant. Recent studies have shown that there are two regions of p53 which can mediate E6 binding in vitro, one within the core domain and one within the carboxy terminus (Li & Coffino, 1996). Which region is bound appears to be influenced by protein concentration and by conformation (Li & Coffino, 1996), and it is likely that both may contribute to the differences observed between the in vitro and in vivo assays. These results clearly demonstrate, however, that the cellular environment can have a profound effect on the susceptibility of a protein to E6-mediated degradation. This conclusion is also supported by a recent study where a hybrid of p53-null peripheral neuroepithelial cells and HeLa cells expressed high levels of p53 protein, despite retaining HPV-18 E6 expression (Isaacs et al., 1997).

At this stage it is difficult to speculate as to what cellular factors might account for the differences between the in vitro and in vivo assays. However, we have also shown that the E1 ubiquitin-activating enzyme is directly involved in the process of E6-mediated degradation of p53 in vivo. Thus, alterations amongst any of the components downstream of the E1 enzyme in the ubiquitin pathway could affect deleteriously the ability of E6 to target p53 for degradation. In addition, there are several reports of extensive post-translational modifications of p53 (Meek, 1994; Milne et al., 1996) but, as yet, no equivalent reports of the HPV-18 E6 protein being modified post-translationally. Thus it is conceivable that these modifications of p53 may alter its susceptibility to E6-induced degradation, thus giving rise to the differences observed between in vitro and in vivo assays. The lack of apparent post-translational modifications for E6 would explain the similarity between the results obtained with the E6 mutants both in vitro and in vivo. It will now be of great interest to determine whether any of the reported post-translational modifications of p53 may alter its susceptibility to E6-mediated degradation in vivo.

In summary, we have performed an extensive analysis of the factors affecting the E6-mediated degradation of p53 in vivo. We have shown that HPV-18 E6 mutants have very similar phenotypes with respect to directing p53 degradation both in vitro and in vivo. In contrast, different forms of p53 display significant differences in susceptibility to E6-mediated
degradation in vitro and in vivo, and these can be influenced by the cell type used. Finally, we have shown that the E1 ubiquitin-activating enzyme is an additional factor which is essential for HPV-18 E6-mediated degradation of p53 in vivo.

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References


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