Characterization of the interactions of human papillomavirus type 16 E6 with p53 and E6-associated protein in insect and human cells

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Human papillomavirus (HPV) 16 E6 induces the degradation of the tumour suppressor protein p53 by the ubiquitin-dependent proteolysis pathway. In vitro, this process involves the formation of a trimolecular complex between E6, p53 and a cellular protein E6-associated protein (E6-AP). However, an analysis of their potential interactions in vivo has not been carried out. We have established a model for the expression and analysis of the interactions of these three proteins in insect cells, a eukaryotic system where potentially crucial modifications of the proteins will occur. In baculovirus-infected cells the degradation of p53 can occur. However, p53 is only degraded early in the infectious cycle due to a lack of ATP at later times. Consequently, substantial quantities of material can be produced in this system for further analysis. Evidence is also provided that, in vivo, E6 can interact with p53 in the absence of E6-AP and that E6-AP can interact with p53 in the absence of E6. Furthermore, analysis of the subcellular localization of the proteins using both biochemical fractionation and indirect immunofluorescence suggests that the degradation of p53 occurs in the perinuclear region of the cell.

Introduction

Human papillomaviruses (HPVs) are involved in the development of cervical cancer (zur Hausen, 1991), since viral DNA can be detected in up to 90% of cervical carcinomas. However, DNA from only a subset of over 70 HPV types is detected in these malignant lesions. Thus HPVs can be further classified as ‘low risk’ types, found primarily in benign lesions, or ‘high risk’ types, found in those lesions that have progressed, or have the potential to progress, to malignancy (zur Hausen & de Villiers, 1994). The transforming properties of the virus reside in ORFs E6 and E7 (Münger et al., 1989; Hawley-Nelson et al., 1989). The E6 protein probably functions by binding to the tumour suppressor protein p53 (Werness et al., 1990) and inducing its degradation via the ubiquitin-dependent proteolysis pathway (Scheffner et al., 1990).

The protein p53 has been classified as ‘the guardian of the genome’ (Lane, 1992) and is normally involved in the regulation of the cell cycle such that, in the event of DNA damage, the cell does not proceed through replicative DNA synthesis but is provided with the opportunity to either repair the damage or die (Martínez et al., 1991; Yonish-Rouach et al., 1991). This prevents potential mutations being passed on to daughter cells. During G1, p53 accumulates in the nucleus (Shaulsky et al., 1990; Martínez et al., 1991) and detects damaged DNA. Following identification of damaged DNA, p53 then activates a set of genes (Zhan et al., 1993) that will inhibit progression through the cell cycle or will stimulate apoptosis (Kastan et al., 1991; Lowe et al., 1993; Clarke et al., 1993).

The ubiquitin degradation pathway involves a cascade of enzymatic events including the ‘activation’ of ubiquitin, its ligation to a protein substrate and, ultimately, the degradation of the ubiquitin–protein substrate (reviewed by Hershko, 1991). A ubiquitin-activating enzyme, E1, catalyses the formation of a thioester between a cysteine residue within itself and the C-terminal glycine of ubiquitin in a process requiring ATP. The ubiquitin is then passed to the thiol site of one of several E2, ubiquitin-conjugating proteins before being transferred to the substrate, often (but not always) in combination with an E3 ubiquitin–protein ligase. Protein–ubiquitin conjugates are produced through the formation of an isopeptide bond between the C-terminal glycine of ubiquitin...
and the ε-amino group of a lysine residue on the target protein. The ubiquitylated protein substrate is subsequently recognized and degraded by the 26S protease complex.

Analysis of the degradation of p53 in vitro established that a third factor was required for the recognition of p53 by E6 and its subsequent ubiquitylation and degradation (Huibregtse et al., 1991). This factor is a cellular protein, E6–associated protein (E6-AP). E6 and E6-AP, in concert, fulfil the role of an E3 ubiquitin–protein ligase (Scheffner et al., 1993). However, although it was originally believed that the E2 carrier proteins catalysed the final ubiquitylation of the substrate protein in conjunction with an E3 protein ligase, the formation of a ubiquitin thioester on E6-AP has set a new precedent for the family of E3s that are the ultimate donors of ubiquitin to the substrate (Scheffner et al., 1995; Rolfe et al., 1995).

The p53 protein is naturally degraded by ubiquitin-dependent proteolysis (Chowdary et al., 1994; Maki et al., 1996) and this process is accelerated in the presence of E6. However, recent evidence suggests that E6-AP is not involved in E6-independent degradation of p53 (Beer-Romero et al., 1997) although it has been demonstrated that E6-AP is capable of stimulating the ubiquitylation of other cellular proteins which have yet to be identified (Scheffner et al., 1993).

Unfortunately, most investigations regarding the interactions of E6, p53 and E6-AP and the consequent degradation of p53 have been carried out in vitro using proteins that have been expressed in a variety of systems, often as fusion proteins. This provides no evidence for the sequence of events in the cell or where in the cell these events occur. The cellular localization of p53 is cell cycle regulated, as already described, but if p53 is unable to be targeted to the nucleus then it is unable to fulfil its growth regulatory function (Shaulsky et al., 1991). The data regarding the localization of E6 are contradictory: it has been localized to the nucleus, the non-nuclear membranes and the cytoplasm (Guio et al., 1989; Grossman et al., 1989; Kanda et al., 1991; Liang et al., 1993). Furthermore, there have to date been no published data regarding the localization of E6-AP.

The aim of the present study was to analyse HPV-16 E6, human p53 and E6-AP and their interactions when all three had been expressed in a model eukaryotic system as non-fusion proteins. The baculovirus/insect cell system was chosen so that significant quantities of eukaryotic expressed material could be obtained for further structural analysis. In addition, insect cells do not have endogenous p53 or E6-AP analogues to complicate the analysis of results, although ubiquitin and the ubiquitin-dependent proteolysis pathway are highly conserved across the species (Jentsch et al., 1991). Even the baculovirus itself encodes a ubiquitin molecule (Guarino, 1990), although its precise role in the life cycle of the virus has yet to be resolved. Consequently, this system was analysed for its potential as a model in which to study the E6-dependent degradation of p53. In addition, the subcellular localization of the three proteins in this system has been ascertained using biochemical fractionation methods and indirect immuno-fluorescence analysis, and these results are related to similar studies using the same antibodies on a panel of human cells with differing E6 content.

**Methods**

**Plasmid constructions.** The p53 cDNA was cloned into the baculovirus transfer vector pVL941 (modified as described by Sanders et al., 1995) by digesting pT7p53 (Daniels et al., 1997) with BamHI and BglII, and ligating the cDNA into pVL941 which had been digested with BamHI and dephosphorylated with calf intestinal phosphatase. The construction of all other clones has been described elsewhere (Daniels et al., 1997).

**Cell culture.** SiHa and HaCaT cells were maintained as monolayer cultures at 37 °C and 5% CO2 in calcium-free 1:1 Dulbecco’s minimum essential medium–Ham’s nutrient mix F-12 supplemented with 10% (v/v) foetal calf serum (FCS) and 2 mM glutamine. The medium for SiHa cells was also supplemented with 1 mM CaCl2. Spodoptera frugiperda SF21 cells were maintained in Grace’s insect cell medium (Gibco-BRL) supplemented with 10% FCS at 27 °C. Cells were routinely adapted from monolayer to spinner culture and were used for the production and propagation of the recombinant baculoviruses. BTI-TN-5B1-4 (Hi5) cells were grown in monolayer under the same conditions as the SF21 cells and were used for the expression of proteins only. Recombinant baculoviruses were generated, purified and propagated according to Sanders et al. (1995) and designated rvE6, rvp53 and rvE6-AP. Hi5 cells were infected at an m.o.i. of 5 or more and maintained at 27 °C for the required time.

**Metabolic labelling of cells and immunoprecipitations.** At 24 h post-infection (p.i.) virus-infected cells were incubated for 1 h at 27 °C in serum-free SF900 II minus methionine and cysteine (Gibco-BRL) before incubating in the methionine/cysteine-free medium supplemented with 70 µCi/ml of Pro-mix (Amersharm) overnight at 27 °C. Cells were harvested at 36 h p.i., after a further 1 h incubation at 27 °C in the presence of an excess of Grace’s medium containing 10% FCS, washed in PBS and then incubated in lysis buffer A (25 mM HEPES pH 7.9, 125 mM NaCl, 0.5% NP-40, 0.1% SDS, 100 µM zinc acetate, 1 mM dithiothreitol, 1 mM PMSF, 1 mM Pepstatin, 10 µg/ml E-64) for 20 min on ice. After centrifugation (9000 g for 20 min at 4 °C), samples of lysate were precleared for 1 h with normal serum and protein A-Sepharose beads.

The lysate was then incubated on ice for 4 h in the presence of p53-specific monoclonal antibody (Ab-1, Oncogene Science) after which the lysate was incubated with 5 µl ubiquitin (2 mg/ml), 10% w/v WGE. Reaction volumes were equated by the addition of WGE and washed three times in an excess of lysis buffer A before analysis by SDS–PAGE and autoradiography.

**p53 Degradation assays.** Insect cells were infected with rvE6, rvE6-AP or wild-type baculovirus. At 48 h p.i. the cells were harvested, washed in PBS, lysed in lysis buffer B (20 mM HEPES pH 7.9, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol) and centrifuged at 50,000 g for 20 min at 4 °C. The total protein concentration of the lysates was measured using the Bio-Rad assay and was subsequently adjusted with lysis buffer B to be equal. Insect cells infected with rvp53 at 24 h p.i. were metabolically labelled and lysed as described above. Assays in the presence of wheat germ extract (WGE) were carried out essentially as described by Daniels et al. (1997), but purified proteins and in vitro translated p53 were substituted for E6 or E6-AP lysates, 1 µl labelled p53 lysate and 10 µl WGE. Reaction volumes were equated by the addition of lysates from wild-type-infected cells. In the absence of WGE, 1 µl p53 lysate was incubated with 5 µl each of the E6 and E6-AP (or wild-type) lysates, 0.5 µl ubiquitin (2 mg/ml), 10 µl 25 mM Tris–HCl pH 7.5,
50 mM NaCl and 1 µl 20 mM ATP, ATP·S or AMPPNP for 4 h at 25 °C. For all degradation assays, any remaining p53 was immunoprecipitated and analysed as described above.

Subcellular fractionation. Subcellular fractionation experiments were carried out as described by Grossman et al. (1989). Fractions were then analysed by SDS–PAGE and immunoblot analysis, essentially as described by Sanders et al. (1995). Production of antisera specific to E6 and E6-AP has been described elsewhere (Daniels et al., 1997) and both were used at a dilution of 1:5000. Supernatants from hybridoma cell lines expressing the monoclonal p53 antibodies pAb421, pAb1620 and pAb240 were used together at dilutions of 1:250.

Indirect immunofluorescence analysis. Human cells were seeded onto poly l-lysine coated slides and incubated until almost confluent. The cells were then washed in PBS, fixed in methanol–acetone (2:1) at room temperature, washed again in PBS and finally in dH₂O before air-drying. Insect cells were seeded on poly l-lysine coated slides, allowed to recover overnight, and infected as described previously. At 24 h p.i. the cells were washed in PBS before fixing in 4% (v/v) formaldehyde in PBS. After washing in PBS, the cells were permeabilized in methanol and then finally washed in dH₂O before allowing to air-dry. All the following steps were carried out at room temperature and all antibodies were diluted in PBS–BSA (3%, w/v, BSA in PBS). Cells were rehydrated in PBS/BSA, blocked in blocking solution (three drops of serum in 10 ml PBS–BSA) and washed twice in PBS–BSA. The primary antibody was then added for 1 h and removed by washing in PBS–BSA. Cells were next incubated for 30 min with secondary FITC-labelled antibodies before washing thoroughly in PBS/BSA. The cells were then mounted under Citifluor (UKC) and coverslips were sealed with clear nail-varnish. Slides were stored at 4 °C before visualizing with a fluorescence microscope.

Results
Expression of E6, p53 and E6-AP in Hi5 insect cells
Hi5 insect cells were infected with one of the recombinant baculoviruses and expression levels were analysed by SDS–PAGE and Coomassie staining. Proteins with the predicted molecular masses for E6, p53 and E6-AP were clearly visible (Fig. 1a). The biochemical activity of the proteins was also determined before any further experiments were carried out. The activities of E6 and E6-AP were measured by their ability to promote the degradation of in vitro translated p53, as previously described by Huibregts et al. (1993) and the biochemical activity of p53 was measured by electrophoretic mobility shift assay of an oligonucleotide containing a specific p53 binding site (data not shown).

Co-expression of E6, p53 and E6-AP
To set up an ex vivo model where the interactions of the three proteins could be analysed, it was necessary to co-express all three proteins. A potential problem was that, as in mammalian cells, p53 would be degraded in the insect cells in the presence of E6 and E6-AP. However, when Hi5 cells were co-infected with the three recombinant baculoviruses, all three proteins were still detectable by Coomassie staining, although the relative level of each protein was severely reduced when compared to protein levels obtained in cells infected with only one baculovirus (Fig. 1b). Their co-expression was unequivocal when the cells were metabolically labelled (Fig. 1c). This suggested that (i) the proteins were unable to interact so as to induce p53 degradation; (ii) they were not being localized to the correct compartment of the cell; or (iii) the infected insect
cells were simply unable to support ubiquitin-dependent proteolysis.

Interactions of E6, p53 and E6-AP expressed in insect cells

To establish that the three proteins were able to interact when co-expressed in insect cells, co-immunoprecipitation experiments were carried out on lysates from cells co-infected with one, two or three of the viruses. As Fig. 2 demonstrates, a p53 monoclonal antibody is capable of co-immunoprecipitating both E6 and E6-AP (lane 6). In addition, E6 alone was co-immunoprecipitated with p53 (lane 4) as was E6-AP alone (lane 5). Identical results were obtained either when lysates from individually infected cells were mixed together and co-immunoprecipitated, or when anti-E6 or anti-E6-AP antibodies were employed (data not shown).

Furthermore, when a metabolically labelled lysate containing p53 was incubated in the presence of E6 and E6-AP lysates and WGE, which is known to provide the necessary components for ubiquitin-dependent degradation to occur (Huibregtse et al., 1991), the p53 was then degraded (Fig. 3). However, in the presence of WGE, degradation of p53 did not take place (Fig. 3b) unless the lysates were supplemented with ATP (Fig. 3c). This was a specific effect of the ATP, demonstrated by the addition of the ATP analogues ATP:S and AMPPNP (Fig. 3d). High molecular mass, presumably ubiquitylated forms of p53 were also evident in the presence of ATP:S (Fig. 3d, lane 5) and, even in the presence of ATP, the degradation of p53 was dependent on the presence of both E6 and E6-AP.

Subcellular localization of the proteins expressed in insect cells

To determine the subcellular localization of the three proteins when expressed in Hi5 cells, fractionation experiments were carried out according to the method of Grossman et al. (1989) and fractions were analysed by Western blotting. At 36 h p.i. all three proteins could be detected in all cellular compartments because of the high levels of expression (data not shown). However, at 24 h p.i., when expressed individually, E6 was localized to the nuclear matrix and non-nuclear membranes and p53 was found mainly in the chromatin fraction with a small proportion associated with the nuclear matrix, but in contrast E6-AP was located primarily in the cytoplasm with some of the protein in the non-nuclear membrane fraction (Fig. 4).

In the light of these results we wished to ascertain whether each of the three proteins affected the subcellular localization of any of the others in the potential complex. When the insect cells were infected with more than one of the baculoviruses, the location of each of the proteins was altered (Fig. 5; summarized in Table 1), although the localization of none of the proteins was completely changed. However, when the insect cells were infected with all three baculoviruses, no p53 was detectable at 24 h p.i. although both E6 and E6-AP were clearly present (Fig. 5; Table 1).

The relatively harsh treatment of cells in fractionation experiments can result in incorrect apparent cellular localizations. Therefore, to confirm the findings of the cellular fractionation experiments indirect immunofluorescence analysis was carried out on the infected insect cells. Unfortunately, Hi5 cells round up and their nuclei are enlarged by 24 h p.i. with the baculoviruses, and this makes distinguishing nuclear, cytoplasmic and membranous compartmentalization very difficult. However, examples of the results are presented in Fig. 6. Cells infected with rvE6, stained with the E6 antibody (Fig. 6a), have a strong nuclear stain which is greater in the outer, membranous region of the nucleus. Cells infected with rvE6-AP, stained with the E6-AP antibody (Fig. 6b), have a halo around the nucleus and weak cytoplasmic staining. An unusual effect was seen in the rvp53-infected cells stained with the p53 antibodies (Fig. 6c) where the nuclear staining was very uneven. It is suggested that p53 is associated with the cellular chromatin which has condensed in the infected cell. In the rvE6/rvp53- and rvE6-AP/rvp53-infected insect cells, the localization of p53 appears to be very similar to that seen before (Fig. 6d, e). However, when the cells were infected with all three viruses no p53 was detected except for small condensed spots towards the outer edge of the cell (Fig. 6f). This was not non-specific background, as these spots were not seen under any other circumstances (data not shown). Cells infected with wild-type Autographa californica multicapsid nuclear polyhedrosis virus (AcMNPV) did not stain with any of the antibodies (data not shown).

Localization of the E6/p53/E6-AP complex in human cells

A similar analysis was carried out on the HPV-16 positive cell line SiHa, to establish that the insect cell model accurately
Fig. 3. In vitro degradation assays. E6 and/or E6-AP lysates, as indicated above the lanes of the gels, were incubated with labelled p53 lysates in (a) the presence or (b–d) the absence of WGE. In (c) the reactions were also supplemented with 1 mM ATP and in (d) the reactions were supplemented with ATP analogues, as indicated above the lanes of the gel.

Fig. 4. Subcellular fractionation of insect cells expressing E6, p53 or E6-AP. After infection with recombinant baculoviruses rvE6, rvp53 or rvE6-AP, Hi5 insect cells were harvested at 24 h p.i. and subjected to subcellular fractionation as described under Methods. Samples of each fraction were then analysed by SDS–PAGE and immunoblotting to locate the protein of interest. (a) rvE6-infected cells; (b) rvp53-infected cells; (c) rvE6-AP-infected cells.

Indirect immunofluorescence analysis provided startlingly clear co-localization of E6 and E6-AP to the perinuclear region of the cell (Fig. 7). As Fig. 7(a) demonstrates, there was still significant background with the E6 antibody in HaCaT cells, but the staining was far more concentrated in SiHa cells, suggesting that the result is specific. In HaCaT cells, p53 has a strong nuclear localization, but is absent from the nucleoli (Fig. 7e), whereas in SiHa cells, as expected, the signal for p53 is much reduced (Fig. 7f). However, there is still some p53 present in SiHa. This is no longer nuclear but occupies the perinuclear region of the cell, in an identical location to E6 and E6-AP.

Discussion

There is only a small amount of in vivo data regarding the E6-dependent degradation of p53. An insect cell model system was set up and analysed in which the interactions of E6, p53 and E6-AP, co-expressed in eukaryotic cells, could be characterized.

Co-immunoprecipitation of the three proteins from co-infected insect cells confirmed that the proteins were able to form a tricomolecular complex ex vivo. Furthermore, the present immunoprecipitation results support the observation of Li & Coffino (1996) that E6 and p53 can interact in an E6-AP-independent manner, and also provide strong evidence for the interaction of E6-AP with p53 in the absence of E6. This latter result is a likely consequence of analysing the proteins when

reflected the situation in human cells. HaCaT, a human keratinocyte cell line, was also analysed as a positive control for p53 expression and as a negative control for E6 expression.
they have all been expressed in a single eukaryotic system as non-fusion proteins. Quantification of the autoradiographs provided no evidence for E6 or E6-AP having a higher binding affinity for p53 if the other protein is present and, when lysates of individually infected cells were mixed together, the same result was obtained as when the proteins were co-expressed (data not shown), indicating that co-translation is not required for the observed interactions to occur.

Degradation assays carried out in the presence of WGE confirmed that the three proteins were able to interact in a manner that would result in p53 degradation. One possible explanation for the lack of p53 proteolysis in infected insect cells is the presence of a baculovirus-encoded ubiquitin homologue, which differs from eukaryotic ubiquitin at 18 residues, in addition to containing an extra C-terminal tyrosine (Guarino, 1990). Although both host and viral ubiquitin are structural proteins of the budded baculovirus (Guarino et al., 1995), viral ubiquitin inhibits ubiquitin-dependent proteolysis in vitro (Haas et al., 1996) which suggests that it probably acts to inhibit the host cell ubiquitin-dependent degradation pathway in vivo. Consequently, the E6/E6-AP-mediated degradation of p53 will also be inhibited. A shortcoming of the in vitro degradation assays is the requirement for supplemented mammalian ubiquitin, which almost certainly overcomes this effect. However, even in the presence of mammalian ubiquitin the lysates do not support p53 degradation unless the reaction is supplemented with ATP. This suggests that the baculovirus-infected cell also lacks the energy required to support this degradation pathway.

On fractionating the cells at 24 h p.i., it was surprising to find that no p53 was detected in the presence of E6 and E6-AP. Because E6 and E6-AP were easily detectable, this observation was unlikely to be due to a lack of translation of p53, but rather was the result of E6/E6-AP-induced degradation of p53. It is therefore proposed that the insect cells have a functional ubiquitin-dependent proteolysis pathway, as do the baculovirus-infected insect cells, up to approximately 24 h p.i. However, this is not the case later in the infection cycle, where ATP and possibly excess viral ubiquitin are limiting factors.

Intracellular locations of baculovirus-expressed recombinant proteins frequently reflect their native locations in eukaryotic cells. Although p53 was degraded in the presence of E6 and E6-AP soon after recombinant virus infection, the cellular localizations of the proteins, when expressed singly, were distinctly different. The localization of E6 to the nuclear matrix and non-nuclear membranes, and of p53 to the nucleus, was in agreement with previous studies in insect cells (Grossman et al., 1989; O'Reilly & Miller, 1988). However, E6-AP was clearly localized to the non-nuclear fractions in the fractionation experiments. This was unexpected, as it had previously been suggested that E6-AP was at least partially nuclear (Huibregtse et al., 1993). In the co-expression analysis, the observed degradation of p53 confirmed that the proteins were functionally interacting, but did not provide a clear indication of where in the cell the complex might be forming.

The ladder of protein species, detected above the main cytoplasmic and non-nuclear membrane E6-AP species in the rvE6/rvE6-AP and rvE6/rvE6-AP co-infected cells but not in the rvp53/rvE6-AP-infected cells (Fig. 5), was previously detected by Huibregtse et al. (1993), who also found that the ladder was dependent on the presence of E6 but not p53. It is likely that this ladder is due to self-ubiquitylation of E6-AP, a process already proposed by Howley and co-workers and also carried out by the hect domain protein RSP5 (Huibregtse et al., 1995).

On breaking the cells during fractionation it is possible for HPV proteins to mislocalize, as seen during the analysis of HPV E7 localization (Smotkin & Wettstein, 1986; Sato et al., 1989). Consequently, indirect immunofluorescence analysis was carried out to confirm the fractionation results. In the presence of E6 and E6-AP, p53 was detected as small,
Table 1. Localization of E6, p53 and E6-AP when co-expressed in insect cells

Results were determined by cellular fractionation and immunoblot analysis as described in the legend to Fig. 4.

<table>
<thead>
<tr>
<th>Cells infected with:</th>
<th>Localization of proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>rvE6 and rvp53</td>
<td>Still mainly localized to the non-nuclear membranes and the nuclear matrix but now also associated with the chromatin</td>
</tr>
<tr>
<td>E6</td>
<td>Present in the non-nuclear membrane fraction as well as the chromatin and nuclear matrix fractions</td>
</tr>
<tr>
<td>p53</td>
<td>Present in the non-nuclear membrane fraction as well as the chromatin and nuclear matrix fractions</td>
</tr>
<tr>
<td>rvE6 and rvE6-AP</td>
<td>Localized to all compartments except the nucleoplasm</td>
</tr>
<tr>
<td>E6</td>
<td>Mainly located in the cytoplasm and the non-nuclear membrane fractions but also some protein associated with the nuclear matrix and the chromatin</td>
</tr>
<tr>
<td>E6-AP</td>
<td>Mainly cytoplasmic with some protein present in the non-nuclear membrane fraction, as when expressed alone</td>
</tr>
<tr>
<td>rvp53 and rvE6-AP</td>
<td>Present in the non-nuclear membrane fraction as well as being associated with the chromatin and the nuclear matrix</td>
</tr>
<tr>
<td>p53</td>
<td>Mainly cytoplasmic with some protein present in the non-nuclear membrane fraction, as when expressed alone</td>
</tr>
<tr>
<td>rVE6, rvp53 and rvE6-AP</td>
<td>Present in all compartments except the nucleoplasm</td>
</tr>
<tr>
<td>E6</td>
<td>No protein observed</td>
</tr>
<tr>
<td>p53</td>
<td>Mainly located in the cytoplasm and the non-nuclear membranes but also associated with the nuclear matrix and the chromatin</td>
</tr>
</tbody>
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condensed spots that were located towards the edge of the cell. These were not an artefact, because they were not seen under any other circumstances, but could represent proteolysis centres in the cell similar to those detected by Wójcik et al. (1996) in HeLa cells. These results suggest that the degradation of p53 occurs outside the nucleus.

Similar immunofluorescence analyses carried out on human cells confirmed the location of E6 and E6-AP as perinuclear; the residual staining of p53 in SiHa cells was also perinuclear. In contrast, p53 in HaCaT cells is mutant (Lehman et al., 1993), which explains the strong nuclear signal for p53 seen in these cells. Analysis of the HPV negative cell line HaCaT indicated that there is some cross-reactivity of the E6 polyclonal antibody against human proteins, as was also observed when analysing total human cell protein by immunoblotting (Daniels et al., 1997). However, the staining of SiHa cells with the various antibodies has such a distinct profile from that of HaCaT cells that the differences are almost certainly due to the presence of E6.

The localization of E6, p53 and E6-AP to the perinuclear region of the cell agrees with that seen for E6 and p53 in other HPV positive cell lines (Liang et al., 1993), as well as with the identification of ubiquitin-mediated proteolysis centres in the same area (Wójcik et al., 1996), and suggests that E6/E6-AP-mediated ubiquitin-dependent p53 degradation occurs in this location. The present observation that the self-ubiquitylated forms of E6-AP are only found in the non-nuclear fractions of the insect cells supports this. Furthermore, a perinuclear localization for E6 places it in the correct site for binding to ERC-55 (or E6-BP), with which E6 also interacts (Chen et al., 1995).

There is also accumulating evidence that p53 is a substrate of the ubiquitin-dependent proteolysis pathway in normal cells (Chowdary et al., 1994; Maki et al., 1996), and the immunoprecipitation results presented here indicate that E6-AP could be the ubiquitin–protein ligase under these circumstances, although recent evidence suggests that this is not the case (Beer-Romero et al., 1997). The papillomaviruses appear to have hijacked this degradation mechanism for their own purposes and the in vitro assays suggest that E6 simply increases the normal rate of p53 degradation. This could occur by several mechanisms: (i) increasing the affinity of E6-AP for p53, although our results do not support this hypothesis; (ii) increasing the reaction rate of the normal reaction pathway; or (iii) using alternative cellular ubiquitylation factors. This latter mechanism has been invoked to explain how E6 continues to mediate the degradation of p53 under conditions where normal p53 ubiquitylation is lost, i.e. when DNA damage has occurred following UV radiation (Maki & Howley, 1997).

Since p53 executes G1 arrest or apoptosis through its functions in the nucleus, then sequestration of p53 outside the nucleus, and degradation in the perinucleus, as proposed here, could also explain this continued degradation of p53. Furthermore, the sequestration of p53 in the cytoplasm, even in
Fig. 6. For legend see facing page.
Fig. 6. Indirect immunofluorescence analysis of insect cells expressing various combinations of E6, p53 and E6-AP. Hi5 insect cells were infected with one or more of the recombinant baculoviruses and were analysed by indirect immunofluorescence analysis using antibodies specific for E6, p53 or E6-AP. (a) Cells infected with rvE6 and analysed with the E6 antibody; (b) cells infected with rvE6-AP and analysed with the E6-AP antibody; (c–f) all analysed with p53 antibodies, cells infected with (c) rvp53; (d) rvE6 and rvp53; (e) rvp53 and rvE6-AP; (f) rvE6, rvp53 and rvE6-AP baculoviruses. The bar below (a) represents 7 £m.

Fig. 7. Immunofluorescence analysis of HaCaT and SiHa cells with the E6, p53 or E6-AP antibodies. (a, c, e) HaCaT cells; (b, d, f) SiHa cells. In each case 1 denotes with primary antibody and 2 denotes without primary antibody; (a, b) E6 antibody; (c, d) E6-AP antibody; (e, f) p53 antibodies. The bar below (a) represents 15 £m.
the absence of its degradation, could provide an additional mechanism by which E6 inhibits p53 function. Li & Coffino (1996) demonstrated that the E6 proteins of the low risk viruses are also capable of interacting with p53 in an E6-AP-independent manner. If one of the mechanisms by which HPV-16 E6 inhibits p53 function is to sequester it in the cytoplasm, then it is likely that E6 from the low risk viruses could operate in a similar manner.

However it is clear from these results and those of others (Grossman et al., 1989; Kanda et al., 1991) that E6 is also able to locate to the nucleus and, in particular, to associate with the nuclear matrix. This is particularly true when E6 is over-expressed in cells. Proteins with a molecular mass of less than 70 kDa are able to move freely between the nucleus and the cytoplasm (Paine et al., 1975). In addition, nuclear localization signals generally constitute a short run of basic amino acids. Therefore it is possible that, being small and highly basic, E6 mistargets to the nucleus when there is a relatively large quantity of the protein in a cell. Alternatively, E6 may have several functions that operate in distinct areas of the cell, like SV40 large T antigen (Butel & Jarvis, 1986), or its localization may even be cell cycle regulated. The localization of some of the other proteins involved in the ubiquitin-dependent pathway is also cell cycle regulated (Amsterdam et al., 1993; Grenfell et al., 1994). Whichever alternative is the case, further work is required to clarify these points, which add sophistication to the already elegant mechanisms developed by HPV for the subversion of cellular processes.

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