Human papillomavirus (HPV) type 18 E7 protein is a short-lived steroid-inducible phosphoprotein in HPV-transformed cell lines

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We used a capture ELISA to quantify the E7 protein of human papillomavirus type 18 (HPV-18). In HeLa cells, which express low levels of immunoreactive E7 protein (iE7), iE7 had a mean half-life of 13.5 min. In HPV-18 E7 recombinant baculovirus (E7rec BV)-infected Sf21 cells, which express higher levels of E7, the half-life of iE7 was much longer (90 min and > 24 h, with two different E7rec BVs). For two transformed human cervical cell lines expressing HPV-18 E7, exposure of the cells to hydrocortisone resulted in a twofold increase in steady-state levels of the E7 protein: no similar effect was observed with progesterone, oestrogen or testosterone. The half-life of iE7 was unaltered by hydrocortisone or progesterone exposure. An immunoassay which distinguished Ser3-phosphorylated E7 from E7 not phosphorylated at this residue (Ser3-dephospho-E7), showed that in HeLa and Sf21 cells the majority of E7 was phosphorylated: the half-life of both species of E7 was similar in HeLa cells, but the half-life of Ser3-dephospho-E7 was much shorter (90 min) in Sf21 cells than that of Ser3-phospho-E7 (> 24 h). A HeLa–fibroblast fusion cell line with tumorigenic potential (CGL-1) had a similar ratio of dephospho-E7 to total E7 (0.06), as a similar fusion cell line (CGL-4) with no tumorigenic potential (0.03). We conclude that E7 is a labile phosphoprotein, and that the expression and steady-state level of the E7 protein in eukaryotic cells may be influenced by the hormonal environment of the cells.

Introduction

Papillomaviruses are dsDNA viruses that infect skin and mucosal surfaces, causing benign tumours. Human papillomaviruses (HPVs), particularly HPV-16 and -18, have been implicated in the aetiology of cervical and other human cancers. The gene products of two early open reading frames (ORFs), E6 and E7, are together sufficient to immortalize human keratinocytes in vitro (Bedell et al., 1987; Barbosa & Schlegel, 1989; Münger et al., 1989a), and expression of the E7 ORF in conjunction with the activated ras gene is sufficient to transform primary epithelial cells to a malignant phenotype (Crook et al., 1989). E7 is thought to disturb cell cycle regulation by binding to the retinoblastoma gene product RB and interfering with the interaction of this protein with other cell cycle regulatory proteins (Münger et al., 1989b). However, studies on the biological activity of the E7 protein have been hampered by the lack of quantitative assays for E7, which is present only in small amounts in transformed cells. The steady-state concentration of E7 protein in cells has generally been inferred from mRNA assays or semi-quantitative immunological techniques (Bosch et al., 1990; von Knebel Doeberitz et al., 1991; Smotkin & Wettstein, 1986; Seedorf et al., 1987). Recently we described a series of monoclonal antibodies (MAbs) specific for the E7 proteins of HPV-16 and -18, and a capture ELISA for the immunoreactive E7 protein (iE7), based on these antibodies (Selvey et al., 1992). We now confirm with this assay that the E7 protein is labile, and have used the assay to investigate the phosphorylation of E7 in E7-transformed cell lines, and the effect of steroid hormones on the half-life and steady-state level of the E7 protein in the transformed cell.

Methods

Cell lines and antibodies. HPV-18-containing cervical carcinoma cell lines HeLa and C4-1 were maintained in RPMI 1640 medium (CSL Laboratories), supplemented with penicillin, streptomycin, t-glutamine (Flow Laboratories), HEPES buffer (Flow Laboratories), sodium pyruvate (Flow Laboratories) and 10% fetal bovine serum (FBS) (CSL Laboratories). The HeLa–human fibroblast hybrid cell lines CGL-1 (non-tumorigenic) and CGL-4 (tumorigenic) (Peelh & Stanbridge, 1981) were a gift from Professor Eric Stanbridge, University of California at Irvine, Ca., U.S.A. The insect cell line Sf21 was a gift from...
Dr David Bishop, Oxford, U.K. and was maintained in TC100 medium with tryptose broth (CSL Laboratories) supplemented with penicillin, streptomycin, sodium bicarbonate (Flow Laboratories) and 10% FBS.

The expression plasmid for the MS2 replicase-HPV-18 E7 fusion protein (MS2/E718) was a gift from Dr Lutz Gissmann. Partial purification of the resultant fusion protein, production of a polyclonal antiserum, and of MAbs, has been described previously (Tindle et al., 1990; Selvey et al., 1990). E7 recombinant baculoviruses were prepared as described (Emery & Bishop, 1987) (Park et al., 1993).

Immunoprecipitation. HeLa cells at 75% confluence in an 80 cm² tissue culture flask were labelled for 1.5 h with 500 μCi of [L-35S]cysteine (Amersham) in cysteine-free DMEM (CSL Laboratories), or 1 mCi of tissue culture flask were labelled for 1.5 h with 500 laCi of L-[35S]cysteine with tryptose broth (CSL Laboratories) supplemented with penicillin, Dr David Bishop, Oxford, U.K. and was maintained in TC 100 medium polyclonal rabbit antiserum against the MS2 replicase-HPV-18 E7 cycloheximide in ethanol (Sigma), or 0.1% ethanol was added to log-

by adding the appropriate volume of cell lysis buffer. For cycloheximide recombinant baculovirus at a high multiplicity were pelleted and lysed aprotinin. Sf21 cells that had been infected 48 h previously with

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expression plasmid for the MS2 replicase-HPV-18 E7 fusion protein. Cell lysates containing the E7 protein of HPV-16 were used as a negative control for the assay. Results were expressed as an estimation of the amount of HPV-18 iE7 concentration was again observed, and the half-life of iE7

Results

We examined the biological half-life of HPV-18 E7 protein in the HPV-transformed cell line HeLa. The concentration of iE7 decayed exponentially with time of exposure to cycloheximide (Fig. 1). The half-life of iE7 was calculated from three experiments to be 130 ± 1.5 min. We then examined the biological half-life of iE7 in Sf21 cell cultures infected with an E7 recombinant baculovirus, vAcl8E7, which gave relatively low-level E7 expression. Initial iE7 in these cells was 227 ng/mg protein, exponential decay of E7 concentration was again observed, and the half-life of iE7

![Graph](https://via.placeholder.com/150)

Two-dimensional (2D) gel analysis of iE7. Cells were suspended in 0.5% NP40 in 150 mm-NaCl, 25 mm-Tris- HCl pH 8.0, 1 mm-MgCl₂ with aprotinin and leupeptin (lysis buffer), passed through a 20-gauge needle and held on ice for 20 min. Lysate was centrifuged at 12000 g for 10 min, and 1 ml supernatant was adjusted to pH 9.0 with 60 μl 1.5 m-

Tris-HCl pH 10. Where appropriate, 250 U alkaline phosphatase (Boehringer Mannheim) was added and the mixture was held at 37 °C for 15 min. Reaction was stopped with 2 μl DTT, and the pH adjusted to 7.5 with 17.5 μl of 2 m-HCl. Lysate was diluted 1:1 with lysis buffer, and E7 was immunoprecipitated after the addition of the appropriate MAb for 2 h at 4 °C, using Protein A-Sepharose beads (Pharmacia).

Beads were washed five times in wash buffer (0.1% NP40 in 150 mm-NaCl, 25 mm-Tris-HCl pH 8.0, 1 mm-MgCl₂ with aprotinin and leupeptin) and incubated for 30 min at 37 °C in isoelectric focusing (IEF) sample buffer. Samples were subjected to equilibrium IEF, PAGE and electroblotting as previously described (Frazier et al., 1985). Blots were blocked with 3% BSA, 0.6% ovalbumin, and exposed to MAbs 15H4 or 20D7 (ascites diluted 1:500 in 3% BSA, 0.6% ovalbumin), washed and bound antibody was detected with horseradish peroxidase-labelled anti-mouse Ig (Kirkegaard) and an enhanced chemiluminescence detection system (Amersham).
was 99 min. We also examined the half-life of iE7 in SF21 cells infected with an E7 recombinant baculovirus, vVL18E7, which produced a high yield of E7 protein. The initial concentration of iE7 was 5735 ng/mg cellular protein, and no measurable loss of iE7 was observed after a 3 h exposure to cycloheximide, giving a calculated half-life for iE7 of > 24 h.

We examined the effect on iE7 concentration in HPV-transformed cell lines of exposure of cells to hydrocortisone and other steroid hormones. Exposure of C4-1 or HeLa cells to hydrocortisone was associated with a consistent increase in iE7. Maximal effect was observed for hydrocortisone doses greater than 0.4 μM in C4-1 cells, and 1 μM in HeLa cells (Fig. 2). The total protein was not altered by exposure to hydrocortisone (Fig. 2). To establish whether there was sufficient hydrocortisone in the FBS used for cell culture to influence the observed results, cell lines were cultured in 2% or 10% FBS prior to E7 assay. For HeLa cells, iE7 was 3.5 ± 0.1 ng/mg protein for cells growing in log phase in 10% FBS, 4.0 ± 0.2 ng/mg for confluent cells in 10% FBS and 4.5 ± 0.6 ng/mg for confluent cells in 2% FBS. Similar results were obtained with C4-1 cells: log-phase cells in 10% FBS had 3.9 ± 0.7 ng iE7/mg protein, similar to confluent cells grown in 10% FBS (3.9 ± 0.4 ng/mg), or confluent cells grown in 2% FBS (3.4 ± 0.3 ng/mg).

Exposure to progesterone, β-oestradiol or testosterone failed to alter iE7 in HeLa or C4-1 cells (Table 1). Corticosterone and progesterone inhibit hydrocortisone receptors with partial agonist activity. Corticosterone at 10 μM slightly increased iE7 in C4-1 cells but had no significant effect on the iE7 concentration in HeLa cells (Table 1). A 10-fold excess of either corticosterone or progesterone showed significant inhibition of the effect of hydrocortisone on iE7. To determine whether the half-life of iE7 was altered in hydrocortisone-treated HeLa cells, they were treated with hydrocortisone and then exposed to cycloheximide. The half-life of the iE7 protein in hydrocortisone-treated HeLa cells (14.7 ± 1.2 min) was not significantly different from that observed with untreated cells (13.0 ± 0.8 min), and we conclude that hydrocortisone increases cellular E7 levels by increasing E7 transcription or translation, rather than by inhibiting breakdown of this protein.

Our E7-specific MAbs were raised against the MS2 replicase–HPV-18 E7 bacterial fusion protein. Most of our E7-specific MAbs immunoprecipitate similar amounts of the HPV-18 E7 protein from lysates of 35S-labelled HeLa cells. In contrast, one MAb, 15H4, which recognizes a six amino acid linear epitope 33SEEEND38

### Table 1. Effect of hormone treatment on immunoreactive HPV-18 E7 protein in HeLa and C4-1 cells

<table>
<thead>
<tr>
<th>Hormone (dose)</th>
<th>iE7 concentration (ng/mg total protein)</th>
</tr>
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<tbody>
<tr>
<td>Ethanol (0.1%)</td>
<td>HeLa: 4.5 ± 0.5* C4-1: 3.8 ± 0.2</td>
</tr>
<tr>
<td>Hydrocortisone (1 μM)</td>
<td>HeLa: 8.5 ± 0.9† C4-1: 7.0 ± 0.3†</td>
</tr>
<tr>
<td>Progesterone (1 μM)</td>
<td>HeLa: 43 ± 0.3‡ C4-1: 40 ± 0.1‡</td>
</tr>
<tr>
<td>Hydrocortisone (1 μM) and progesterone (10 μM)</td>
<td>HeLa: 54 ± 0.6§ C4-1: 56 ± 0.4§</td>
</tr>
<tr>
<td>β-Oestradiol (1 μM)</td>
<td>HeLa: 41 ± 0.4∥ C4-1: 44 ± 0.4∥</td>
</tr>
<tr>
<td>Testosterone (1 μM)</td>
<td>HeLa: 47 ± 0.4† C4-1: 40 ± 0.1†</td>
</tr>
<tr>
<td>Corticosterone (10 μM)</td>
<td>HeLa: 40 ± 0.2</td>
</tr>
<tr>
<td>Hydrocortisone (1 μM) and corticosterone (10 μM)</td>
<td>HeLa: 45 ± 0.4</td>
</tr>
</tbody>
</table>

* The number of independent variables (n) and the S.E.M. are shown.
† P < 0.005 compared with ethanol (Student's t-test).
‡ P not significant compared with ethanol.
§ P < 0.05 compared with hydrocortisone.
∥ P < 0.05 compared with ethanol.
of E7 (Selvey et al., 1990) including one of three potential serine phosphorylation sites in this protein, immunoprecipitates only trace amounts of E7 protein from lysates of $[^{35}S]$cysteine-labelled HeLa cells, although recognizing E7 produced in a prokaryotic expression system as effectively as the other E7-specific MAbs. When $^{35}$S-labelled HeLa cell lysates were treated with alkaline phosphatase, and then subjected to immunoprecipitation with 15H4, a more intense band with an appropriate size for E7 could be identified (data not shown). We examined further the specificity of 15H4 for HPV-18 E7, using 2D analysis of E7 protein immunoprecipitated from Sf21 cells overexpressing this protein after infection with vVL18E7 (Fig. 3). E7 protein precipitated by 17C6 was detected by 17C6 as a series of proteins with isoelectric points from 5.5 to 9 (Fig. 3a), whereas the E7 protein species detected by 15H4 were concentrated at lower $pK_a$ values (Fig. 3b). Treatment of cell lysates with alkaline phosphatase prior to immunoprecipitation increased the total amount of E7 protein precipitated by 15H4 (Fig. 3d), although not the total amount of E7 precipitated by another E7-specific MAb 17C6 which recognizes a conformational epitope (Fig. 3c). 2D gel analysis of the E7 species precipitated by MAb 17C6 showed a continuous rather than a discrete series of E7 proteins: the single species recognized by 15H4 corresponded to the most acidic of these and was not shifted in electrophoretic mobility by alkaline phosphatase treatment. Taken together, these data suggest that 15C4 recognizes only the E7 species not phosphorylated at Ser$^{33}$, and that, in HeLa cells and Sf21 cells overexpressing E7, the vast majority of E7 which is not phosphorylated at this residue is totally dephosphorylated. 2D gel analysis of immunoprecipitated E7 protein by MAb 17C6 shows a marked heterogeneity of E7 species, not reduced by alkaline phosphatase treatment. Presumably the more basic E7 species represent post-translational modifications of E7 other than phosphorylation, and could be precipitated from the cell lysate by MAb 15H4 only after treatment of lysate with alkaline phosphatase, suggesting that these modified E7 species are also routinely phosphorylated.

To investigate the phosphorylation at Ser$^{33}$ of E7 expressed in cervical cancer cells, 15H4 was used as a capture antibody for the detection of HPV-18 E7 protein in HeLa cells, using a sandwich ELISA standardized against a constant amount of a MS2 replicase–HPV-18 E7 bacterial fusion protein, which is not phosphorylated. When iE7 was determined in HeLa cell lysates, an 15H4-based ELISA gave a ratio for the reactivity of HeLa cell lysates to that of MS2 replicase–HPV-18 E7 fusion protein which was 10-fold lower than when iE7 was determined using 7E10, or any of the other HPV-18 E7-specific MAbs (Table 2). 15H4 therefore reacts with an
Table 2. Estimation of HPV-18 E7 protein concentrations in a sandwich ELISA using different MAbs as the capture layer

<table>
<thead>
<tr>
<th>MAb*</th>
<th>Epitope†</th>
<th>A492 with HPV-18 E7‡</th>
<th>MAb: 7E10 ratio§</th>
</tr>
</thead>
<tbody>
<tr>
<td>13D12</td>
<td>A</td>
<td>0·580</td>
<td>2·2</td>
</tr>
<tr>
<td>15H4</td>
<td>B</td>
<td>1·54†</td>
<td>0·06</td>
</tr>
<tr>
<td>7E10</td>
<td>C</td>
<td>0·900</td>
<td>1</td>
</tr>
<tr>
<td>17C6</td>
<td>Conf1</td>
<td>0·600</td>
<td>1·3</td>
</tr>
<tr>
<td>17A6</td>
<td>Conf2</td>
<td>0·138</td>
<td>2·0</td>
</tr>
</tbody>
</table>

* MAb used as the capture layer.
† Epitopes were defined with a series of overlapping octapeptides spanning the predicted amino acid sequence of HPV-18 E7 (Selvey et al., 1990). Conf1 and Conf2 are presumed conformational epitopes, defined by the pattern of reactivity of the MAbs in competition assays.
‡ The A492 values were obtained in the sandwich ELISA using 1·5 ng of MS2 replicase-HPV-18 E7 fusion protein as the substrate.
§ Estimated HPV-18 E7 protein concentration in a HeLa cell extract, determined by ELISA using the MAb under test as the capture antibody, expressed as a ratio to the HPV-18 E7 protein concentration in the same extract, estimated by ELISA using MAb 7E10.

Fig. 4. iE7 detected by two MAbs in vVL18E7-infected Sf21 cells before and after treatment of cell lysate with alkaline phosphatase. Cell lysates were exposed to alkaline phosphatase for 0, 0·1, 2 and 10 min, the reaction was stopped with DTT, and the lysates were tested by capture ELISA for iE7 using MAb 15H4 (○) or 7E10 (□). Untreated cell lysates were assayed in parallel (data not shown). Results are the means ± 1 S.D. of four determinations.

Table 3. Ratio of non-phosphorylated to total HPV-18 E7 protein in various cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Non-phosphorylated iE7:total iE7 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0·06</td>
</tr>
<tr>
<td>CGL-1</td>
<td>0·06</td>
</tr>
<tr>
<td>CGL-4</td>
<td>0·03</td>
</tr>
<tr>
<td>C4-1</td>
<td>0·1</td>
</tr>
<tr>
<td>vVL18E7-infected Sf21</td>
<td>0·006</td>
</tr>
</tbody>
</table>

Discussion

The short half-life of the HPV-18 E7 protein which we have demonstrated in eukaryotic cells is consistent with that observed for other regulatory proteins (Luscher & Eisenman, 1988; Ron et al., 1989; Schubach & Horvath, 1988), and is in keeping with previously reported observations of the half-life of HPV-16 E7 protein in cervical carcinoma cells (Smotkin & Wettstein, 1987). The prolonged half-life of iE7 expressed in insect cells was not predicted, as the half-life of mammalian proteins expressed in insect cells is generally similar to that in mammalian cells (Tratner et al., 1990; Grossman et al., 1989). The longer half-life in insect cells of HPV-18 E7 protein expressed by vVL18E7 compared to vAc18E7 suggests that, at least in these cells, the E7 half-life is proportional to the initial E7 concentration, and activity of the enzyme(s) required for degradation of HPV-18 E7 protein may be limiting the rate of degradation.

The phosphorylation state of many proteins involved in cell cycle regulation is critical to their biological activity. HPV-18 E7 has three potential phosphorylation sites, including Ser33, which is included in the linear epitope recognized by MAb 15H4 (Selvey et al., 1990), and the data presented here suggest that the majority of HPV-18 E7 protein in HeLa cells is phosphorylated at
Ser<sup>22</sup>; Ser<sup>33</sup> in HPV-18 E7 protein corresponds to Ser<sup>22</sup> of the HPV-16 E7 protein. Immunoprecipitation from <sup>32</sup>P-labelled CaSkis cells with a rabbit polyclonal antiserum specific for HPV-16 E7 protein has shown that at least a portion of immunoreactive HPV-16 E7 protein is phosphorylated, at at least one serine residue (Smotkin & Wettstein, 1987), and double substitutions of serine residues 31 and 32 appeared to prevent all phosphorylation of the E7 protein (Barbosa et al., 1990); hence these residues may be the only significant sites of E7 phosphorylation. Our data showed no evidence of differences in E7 protein phosphorylation between two E7-expressing transformed HeLa–fibroblast cell lines of different tumorigenic potential, which appears to exclude regulation of E7 phosphorylation as the mechanism by which the different cell growth properties of these lines occurs.

Point measurements of iE7 protein concentration in response to external stimuli are unable to differentiate between changes in synthesis, in catabolism or in immunoreactivity of E7 protein in cell lysates. A dexamethasone-induced increase of HPV-16 and -18 mRNA levels in cervical cancer cell lines has been reported (Chan et al., 1989; von Knebel Doeberitz et al., 1988, 1990). A previous report on HPV-18 E7 protein used a semi-quantitative assay based on a polyclonal antiserum (von Knebel Doeberitz et al., 1988, 1990) to show a glucocorticoid-associated increase in immunoreactive HPV-18 E7 protein in C4-I cell lysates. Our quantitative E7 assay has allowed us to show that the steroid-induced increase in E7 is dose-related, is unrelated to steroid-induced changes in total cellular protein concentrations and occurs over the physiological range of concentrations of hydrocortisone. A postulated mechanism for this increase is via a glucocorticoid response element in the long control regions of both HPV-16 and -18 (Chan et al., 1989; Chong et al., 1990). The extremely short half-life of HPV-18 immunoreactive E7 protein suggested that the rate of catabolism of the E7 protein could also be a major determinant of immunoreactive E7 protein levels, but we did not observe a change of the half-life of HPV-18 E7 protein in response to hydrocortisone. Some glucocorticoid response elements are also responsive to progesterone, when progesterone receptors are present in the cell (Strähle et al., 1990), whereas oestrogen response elements are related but distinct (Klock et al., 1987). The levels of progesterone and oestrogen receptors in HeLa and C4-I cell lysates were determined by radioimmunoassay and no oestrogen or progesterone receptors were detectable in either cell line (data not shown).

Levels of E7 protein appear to influence the proliferative behaviour of HPV-transformed cells (von Knebel Doeberitz et al., 1990). Progesterone or dexamethasone is a necessary cofactor for the transformation of primary baby rat kidney cells by the early region of HPV-16 or -18 DNA and activated ras, when expression of the HPV early region is not driven by a strong heterologous promoter (Pater et al., 1988, 1990). In vitro, hydrocortisone also increases the frequency of transformation of human keratinocytes by HPV-16 DNA (Schlegel et al., 1988). As hydrocortisone and progesterone interact with a common glucocorticoid response element, a demonstration of a similar effect of progesterone on immunoreactive E7 protein levels in cervical epithelial cells, which are known to possess a progesterone receptor, could contribute to the higher incidence of invasive cervical cancer in long-term users of the oral contraceptive pill, and the increased detection of HPV DNA in cervical samples from women in the third trimester of pregnancy (Schneider et al., 1987; Rando et al., 1989).

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References


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