The Major Human Papillomavirus Protein in Cervical Cancers Is a Cytoplasmic Phosphoprotein

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In a previous study, the most abundant viral transcript in a human papillomavirus type 16-associated cervical cancer and in a cancer-derived cell line was characterized, and its translation product, the E7 protein, was identified (D. Smotkin and F. O. Wettstein, *Proc. Natl. Acad. Sci. USA* 68:4680-4684, 1986). Here we show that the E7 protein had a half life of about 1 h and was located in the soluble cytoplasmic fraction. The protein was phosphorylated at serine residues and exhibited a high heterogeneous sedimentation rate in nondenaturing glycerol gradients, suggesting an oligomer formation or association with cellular protein.

A high percentage of cervical cancers contain human papillomavirus (HPV) DNA sequences; most frequently, it is HPV type 16 (HPV-16) (2, 4, 9, 11, 17). We have mapped the HPV-16 transcripts in a cervical cancer and a cancer-derived cell line (18). In both, the most abundant transcript codes for the early protein E7. We have identified the E7 protein in two cancer-derived cell lines. Additional transcripts could code for E6, and in the cancer additional transcripts could also code for E2 (18). The role established for E7 by genetic experiments with bovine papillomavirus type 1 (BPV-1) is to maintain papillomavirus plasmid DNA at a high copy number in transformed cells (1, 8). This role would not appear relevant in cervical cancers, which most frequently have only integrated HPV DNA. To investigate possible functions of E7 in cancer development, we have studied biochemical properties of the protein in two cancer-derived cell lines.

The first property we investigated was the metabolic stability of E7 as determined by pulse-chase experiments. For those experiments, CaSki and SiHa cells were grown in RPMI medium 1640 with 10% and 15% fetal bovine serum, respectively, and supplemented with penicillin and streptomycin. Subconfluent cultures in 60-mm dishes were refed the day before labeling. Plates were rinsed twice with RPMI medium 1640 lacking methionine and cysteine and incubated for 30 min with 2 ml of medium without cysteine and containing 10 μ M methionine and 10% dialyzed fetal bovine serum. L-[³⁵S]cysteine (0.5 mCi) was added, and the incubation was continued for 1 h. One culture was harvested directly, and in the others, the labeled medium was replaced by unlabeled medium and additional cultures were harvested after a 1-, 2-, or 4-h chase. The cultures were rinsed three times with Tris-buffered saline, and the cells were removed in 0.5 ml of RIPA buffer. The immune precipitation of E7 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis were as described (18).

As can be seen with CaSki and SiHa cells, the intensity of the E7 band decreased rather quickly (Fig. 1). Autoradiograms obtained after different times of exposure were scanned with a recording and integrating spectrophotometer. The value calculated for the half-life was 55 min for the CaSki cells and 70 min for the SiHa cells. The short half-life may be indicative of a regulatory function of the protein. Furthermore, there was no change in mobility detectable

during the chase, which suggests that there were no major modifications such as an extensive glycosylation or proteolytic cleavage.

The subcellular location of E7 was determined by cell fractionation. CaSki cells were labeled for 1 h with L-[³⁵S]cysteine as described above. The cells were harvested by trypsinization, suspended in 1 mM KCl-0.2 mM MgCl₂-4 mM Tris hydrochloride (pH 7.6)-0.2 mM phenylmethylsulfonyl fluoride, and broken by homogenization in a tight-fitting Dounce homogenizer. A crude nuclear fraction was obtained by sedimentation for 5 min at 250 $\times$ g in a Sorvall RC-3 centrifuge. The supernatant was further separated into a membrane and soluble cytoplasmic fraction by centrifugation in 2-ml tubes in a 50 Ti rotor (Beckman) for 30 min at 81,000 $\times$ g. Membrane and nuclear fractions were directly dissolved in RIPA buffer and immunoprecipitated as described for the pulse-chase experiments. The soluble cytoplasmic fraction was brought to 0.1 M KCl, 0.005 M MgCl₂, and 0.01 M Tris hydrochloride (pH 7.6) by the addition of 1/9 volume of a 10 $\times$ solution. RIPA buffer (1/9 volume; 10 $\times$) was then added, and immunoprecipitation of E7 was performed as described for the nuclear and membrane fractions.

The electrophoretic analysis of immunoprecipitates shows that E7 is only present in the high-speed supernatant (Fig. 2). When considering the genetically defined function of E7 in BPV-1 (1, 8), the presence of E7 in the soluble cytoplasmic fraction is rather unexpected. It is of course possible that the function of E7 in plasmid maintenance is an indirect one not requiring its presence in the nucleus, or only trace amounts of the protein may be required directly and this could have escaped detection. Furthermore, there may be differences in E7 function between HPV-16 and BPV-1. Certainly there are differences in the parent transcripts. BPV-1-transformed cells contain a transcript coding for an E6-E7 fusion protein (20, 22). No transcript for such a fusion protein was detected in CaSki cells, nor have such transcripts been detected in tumors induced by the cottontail rabbit papillomavirus (3, 5, 10). It may be significant that the frameshift mutation in BPV-1 resulting in a low plasmid copy number would affect both an E6-E7 fusion protein as well as an E7 protein, and the cDNA complementing this mutation was derived from an mRNA coding for an E6-E7 fusion protein (1, 8).

Labeling of CaSki cells with ³²P₄ permitted the detection of a cytoplasmic phosphoprotein which comigrated with

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FIG. 1. Metabolic stability of the HPV-16 E7 protein in CaSki and SiHa cells. The E7 protein was immunoprecipitated from extracts prepared from cells labeled for 1 h, chased for different time periods, and analyzed by SDS-PAGE. ^{14}C -labeled molecular weight standards were run in lane S, and their size in kDa is indicated on the right. The position of E7 is indicated by the arrow. Lanes: 0, 1-h label; 1, 2, and 4, 1-h label followed by a 1-, 2-, or 4-h chase, respectively.

^{35}S -labeled E7 (Fig. 3A). In these experiments, CaSki cells were grown as described for Fig. 1. Cultures were rinsed twice with Tris-buffered saline and preincubated for 15 min with 1 ml of phosphate-free RPMI medium 1640 containing 10% dialyzed fetal bovine serum. $^{32}\text{PO}_4$ was then added (0.5 mCi per dish), and the incubation was continued for 1 h. Cells were harvested by trypsinization and washed twice

with Tris-buffered saline. The cell pellet was suspended in 0.01 M NaCl–0.01 M Tris hydrochloride (pH 7.6)–0.0015 M MgCl_2 –0.5% deoxycholate–1% Nonidet P-40–0.2 mM phenylmethylsulfonyl fluoride and briefly vortexed. Nuclei were pelleted by centrifugation, and E7 was immunoprecipitated from the cytoplasmic fraction and analyzed by gel electrophoresis as described for Fig. 3.

The identity of the ^{32}P -labeled protein with E7 was further suggested by blocking experiments. When antisera were first treated with *Escherichia coli*-synthesized trp E-E7 fusion protein, it prevented the immunoprecipitation of the ^{32}P -labeled band (Fig. 3, lane 1), and this also prevented the precipitation of ^{35}S -labeled E7 (18). Furthermore, when CaSki cells were labeled with $^{32}\text{PO}_4$ for 1.5 and 3 h, the intensity of the band did not increase while other bands increased in intensity, indicating that the ^{32}P -labeled protein also had a short half-life (not shown). A band migrating slightly faster than E7 and visible as a faint band in lane 1 (Fig. 3A) had the same intensity as E7 after 3 h of labeling. However, coprecipitation of this band was nonspecific since it was not inhibited by blocking of the antiserum with the trp E-E7 fusion protein.

To determine the identity of the ^{32}P -labeled amino acid(s), E7 was located in acrylamide gels by direct autoradiography, and the band was cutout from multiple lanes. E7 was extracted from the gel and hydrolyzed (7, 23) in the presence of a carrier mixture consisting of bovine serum albumin (BSA), ovalbumin, and lysozyme and which also contained phosphoserine, phosphothreonine, and phosphotyrosine. The phosphoamino acids were separated by paper electrophoresis (7, 23). The analysis revealed only the presence of phosphoserine among the three potential phosphorylated amino acids (Fig. 3B). Our data thus show that E7 is a phosphoprotein. It shares this property with large T antigen of simian virus 40 (21) and the large and middle T antigens of polyomavirus (12).

FIG. 2. E7 is a cytoplasmic protein. A detergent-free lysate of CaSki cells labeled for 1 h with ^{35}S -cysteine was separated into a cytoplasmic high-speed supernatant, a membrane fraction, and a nuclear fraction. Electrophoresis and autoradiography were as described for Fig. 1. S, Molecular weight standards; C, soluble cytoplasmic fraction; M, membrane fraction; N, crude nuclear fraction. The position of E7 is indicated by the arrow.

FIG. 3. E7 is a phosphoprotein-containing phosphoserine. (A) SDS-PAGE analysis of ^{32}P -labeled protein from CaSki cells. Lane S, Molecular weight standards; lane 1, immunoprecipitation of a $^{32}\text{PO}_4$ -labeled cytoplasmic extract with antiserum blocked with tryp E-E7 fusion protein; lane 2, same as lane 1, but with unblocked antiserum; lane 3, same as lane 2 except cells were labeled with L- ^{35}S -cysteine. The position of E7 is indicated by the arrow. (B) High-voltage paper electrophoresis of the hydrolyzed E7 protein. Lane 1, Autoradiogram; lane 2, ninhydrin staining.

FIG. 4. Sedimentation analysis of E7. ^{35}S -labeled cytoplasmic extracts were sedimented on glycerol gradients. (a) The extract and the gradient did not contain detergents. (b) The extract was prepared in RIPA buffer, and the gradient contained 0.5% Triton X-100. (c) Immunoprecipitated E7 was released from protein A-Sepharose and sedimented in a gradient containing 0.1% lithium dodecyl sulfate. Lanes 11 and 13 in each gradient contain half the fractions 11 and 12, and 13 and 14, respectively. Molecular weight standards (lysozyme, ovalbumin, BSA, and immunoglobulin G) were sedimented in parallel gradients, and the position of the protein was determined by scanning gradients at 280 nm. The positions of lysozyme (13 kDa) and BSA (68 kDa) in each gradient are indicated by the vertical arrowheads. The sedimentation rate of lysozyme was the same in all gradients. BSA sedimented slightly more slowly in panel c. The horizontal arrowheads indicate the position of the E7 protein. The positions of molecular weight standards in the SDS-PAGE are as given in panel a.

A potential association of E7 with cellular proteins was investigated by glycerol gradient analysis (Fig. 4). Soluble cytoplasmic extracts were prepared as described for Fig. 2. The salt concentration was adjusted to 0.1 M KCl, 0.005 M MgCl_2 , and 0.05 M Tris hydrochloride (pH 7.6), and the extracts were sedimented in 5 to 20% glycerol gradients at 4°C for 16 h at $190,000 \times g$ in an SW50.1 rotor (Beckman). The sedimentation in Fig. 4a was in 0.1 M KCl–0.005 M MgCl_2 –0.05 M Tris hydrochloride (pH 7.6). In Fig. 4b, an extract prepared as in panel a was supplemented with 1/9 volume of $10\times$ RIPA buffer and sedimented in a gradient which also contained 0.5% Triton X-100. Finally, for Fig. 4c, E7 was immunoprecipitated from a soluble cytoplasmic extract, released from protein A-Sepharose as described for Fig. 1 except that lithium dodecyl sulfate replaced SDS and sedimented in a gradient containing 0.1 M LiCl, 0.05 M Tris hydrochloride (pH 7.6), and 0.1% lithium dodecyl sulfate.

Fractions of 0.33 ml were collected from the top of the gradients. For Fig. 4a and b the fractions were immunoprecipitated and analyzed as described for Fig. 1. A

portion of each fraction was directly analyzed by SDS-PAGE (Fig. 4c). The slow migration of E7 in SDS-PAGE corresponds to a protein of about 20 kilodaltons (kDa) (Fig. 2) rather than the 11 kDa calculated from the sequence (16), and suggested some unusual properties. This was confirmed by sedimentation in denaturing glycerol gradients (Fig. 4c), where E7 was seen to sediment slower than lysozyme. Indeed, the sedimentation rate, although not very accurate for a slowly sedimenting protein under the assay conditions, would be that of a protein about half the size of lysozyme. A similar disparity between migration in SDS-PAGE and sedimentation in glycerol gradients has also been described for the E1A protein of adenovirus type 2, for which an unusual extended structure was proposed (19).

When sedimented on a nondenaturing gradient (Fig. 4a), E7 was dispersed over a wide range. About one-third of E7 appeared to sediment faster than BSA, and very little sedimented more slowly than lysozyme. In parallel gradients, the marker proteins lysozyme, ovalbumin, BSA, and immunoglobulin G sedimented as monodisperse peaks (not shown). The position of the lysozyme and BSA markers is indicated in the figure. The sedimentation of E7 in Fig. 4b is intermediate between that in panels a and c. For Fig. 4b the cytoplasmic extract was treated with RIPA buffer before sedimentation on a gradient containing the nonionic detergent Triton X-100. The high sedimentation rate in detergent-free gradients could indicate that E7 forms oligomers or that it associates with another protein(s). Unambiguous identification of such a protein is not possible at present. It would require antisera, preferably monoclonal antibodies, against such a protein.

Less compelling evidence can be adduced from the cosedimentation of other protein(s) with E7. Based on this criterion, one protein with a mobility slightly higher than that of the 46-kDa marker could be a potential candidate. First, in nondenaturing gradients this protein had a distribution similar to E7 (Fig. 4a) except it was not present in fractions 2 and 3; this could suggest that some E7 was free in monomeric form. Second, when a cytoplasmic extract was treated with RIPA buffer before gradient analysis, there was a similar shift in the sedimentation of this protein (not shown) and E7 (Fig. 4b). In this regard, this protein and E7 behave similarly to a 61-kDa protein which appears to interact with middle T antigen of polyomavirus and for which RIPA buffer causes a shift in the sedimentation of the complex (6).

Genetic analysis of BPV-1 permitted assignment of the transforming activity to E6 (13, 22), whereas E7 or an E6-E7 fusion protein is required to maintain viral DNA plasmids at a high copy number (1, 8). Based on these results, one would not anticipate finding mRNA coding for E7 as the most abundant transcript in HPV-16 DNA-containing cervical cancers, which most often have only integrated viral DNA. There are several interpretations possible to reconcile these different observations. First, and least likely, it is possible that only continued expression of E6 is required in cancers and the presence of E7 is fortuitous since its mRNA is generated from the same primary transcript as that for E6 (18). Evidence against this interpretation can be adduced from the analysis of HPV 18-containing cervical cancer-derived cell lines (15). Not all of these cell lines contain the whole viral genome, but the region coding for E6 and E7 is always preserved, and again the viral transcripts identified can code for E6 and E7 (14). Furthermore, with cottontail rabbit papillomavirus, a virus which also causes malignant tumors in its natural host, the mRNAs for E6 and E7 are generated from different primary transcripts (3, 5, 10). Nev-

ertheless, there is a tendency that E7 transcripts are more abundant relative to E6 transcripts in cancers than in benign papillomas (10), and this is most evident in the VX-2 transplantable cottontail rabbit papillomavirus-induced carcinoma, which contains only integrated viral DNA (5).

Second, there could exist differences in some of the viral protein functions between different papillomaviruses. Thus, only some are associated with malignant tumors, among them HPV-16. In addition, as indicated, a transcript for an E6-E7 fusion protein has only been identified for BPV-1 (20, 22).

Finally, and we consider this most likely, viral functions required for transformation of cells in culture may be overlapping with, but may not be identical to, those required for progression to and maintenance of cancers in a natural host. Thus E6 of HPV-16 may be able to transform cells in culture in the absence of E7, but E7 may be required in addition to E6 for progression to a malignant state in the natural host.

The cytoplasmic location of the protein suggests that its function in plasmid maintenance is an indirect one and E7 could have a pleiotropic effect. To elucidate a potentially complex function of E7, it will be important to further characterize its biochemical properties, to identify the kinase(s) involved in its phosphorylation, and to establish any interaction with cellular protein(s) or its oligomerization.

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