

Functional oligomerization of purified human papillomavirus types 16 and 6b E7 proteins expressed in *Escherichia coli*

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Purified non-fused soluble human papillomavirus type 16 and 6b E7 proteins expressed in *Escherichia coli* were found to form oligomers. For both proteins, several degrees of oligomerization were demonstrated by gel filtration, dynamic laser light scattering and scanning

electron microscopy. Oligomerization was dependent on the concentration of E7 protein. Oligomerized E7 proteins were able to bind the retinoblastoma gene product pRB and stimulated DNA synthesis when introduced into cells.

Introduction

More than 60 types of human papillomavirus (HPV) have been identified to date and are known to cause various epithelial lesions (de Villiers, 1989). The HPVs affecting genital mucosa can be divided into two groups: the high and low risk groups. The E7 gene product of HPV type 16, which belongs to the high-risk group, is considered to play an important role in cellular transformation (Yasumoto *et al.*, 1986; DiMaio, 1991) and carcinogenesis (zur Hausen, 1983; Yee *et al.*, 1985; Campo & Jarrett, 1987). Regarding the mechanism, binding of the E7 protein with pRB, the product of the tumour suppressing retinoblastoma gene, is considered to be a major factor in transformation (Dyson *et al.*, 1989; Munger *et al.*, 1989). In contrast, HPV type 6b, which belongs to the low risk group, is associated with condylomata acuminata, and the type 6b E7 protein shows a lower affinity for pRB than that of type 16 (Heck *et al.*, 1992). Although both types of E7 protein are composed of 98 amino acids and share 52% identity, their behaviour in SDS-PAGE differs, perhaps due to a conformational difference (Gage *et al.*, 1990; Armstrong, 1992). It is not known whether this is related to the different tumorigenicities of the two viruses. Thus, we compared the physicochemical characteristics of the purified E7 proteins of HPV types 16 and 6b expressed in *Escherichia coli*. We found that E7 proteins from both types tend to form oligomers. This finding agrees with and extends a recent report (McIntyre *et al.*, 1993) that HPV-18 E7 protein forms dimers and oligomers through the zinc finger motif, which is common to the E7 proteins of HPV types 16, 18 and 6b.

Methods

Plasmids and vectors for HPV E7 protein expression. HPV type 16, 16 mutant (24C-G) and 6b E7 proteins were produced in *E. coli* strain BL21(DE3)pLysS (Studier & Moffatt, 1986). A plasmid producing HPV-16 mutant (24C-G) E7 protein, in which the Cys at position 24 from the N terminus that is critical for pRB binding (Y. Matsushima & M. Terada, unpublished) was substituted by Gly, was created by site-directed mutagenesis from pMXE7 (Imai *et al.*, 1991). A plasmid, pMX6E7, that expresses HPV-6b E7 protein, was created as follows. A pair of sense and antisense primers, AATTGTACGTGGAAGG-GTCG and GAATCGTCCGCCATGGTTGTTAGGTCTTC, were synthesized, whose sequences contain *Eco*T22 and *Nco*I sites, respectively. The antisense primer sequence had a mismatch of one nucleotide (C-G) at position 15 from the 5' end of the HPV-6b gene (de Villiers *et al.*, 1981). Using the primers, a PCR was carried out with Amplitaq (Cetus) in the presence of a template DNA of pPH6b (Schwartz *et al.*, 1983) containing the full HPV-6b sequence, by cycling 35 times at 94 °C for 1.5 min, at 50 °C for 2 min and at 68 °C for 2 min. The PCR product was electrophoresed in a 3% agarose gel and a DNA band at 300 bp was extracted. The DNA fragment was ligated with a vector (which had been excised with *Eco*T22 and *Nco*I from pMXE7) to form the plasmid pMX6E7. BL21(DE3)pLysS cells were transformed with pMX6E7. The subcloned 300 bp of plasmid DNA in one of colonies was sequenced and found to match completely the E7 gene sequence in pPH6b (sequenced by Takara Custom Service). The *E. coli* cells harbouring pMXE7 and pMX6E7 were cultured in the presence of 100 µg/ml ampicillin and 10 µg/ml of chloramphenicol, and protein production was induced with 0.5 mM-IPTG and 1 mM-zinc acetate.

Purification of HPV E7 proteins. Crude extracts from bacterial cells in 50 mM-Tris-HCl pH 8.5 containing 5% glycerol, 1 mM-PMSF and 1 mM-DTT were loaded on high load Q-Sepharose (Pharmacia-LKB) and eluted with buffer A (10 mM-HEPES pH 7.5, 1 mM-DTT, 5% glycerol, 20 µM-zinc acetate) containing a NaCl gradient ranging from 0.2 to 1 M. Peak fractions containing the E7 proteins, as confirmed by SDS-PAGE and Western blotting, were concentrated by ultrafiltration using Diaflo YM5 (Amicon) and applied to Superdex G-75 (Pharmacia-LKB). Protein samples were dialysed against 5 mM-HEPES buffer pH 7.5 containing 20 µM-zinc acetate and used for all the following

experiments. The N-terminal amino acid sequence (MHGRH---) of purified HPV-6b E7 protein was confirmed (sequenced by Takara Custom Service).

pRB expression and purification. pRB was produced in *E. coli* harbouring pGEX2T-RB(379-928) (Kaelin *et al.*, 1992) and was purified on glutathione-Sepharose following Kaelin's methods.

Dynamic laser light scattering (DLS). The hydrodynamic radii of protein clusters were measured using a DLS apparatus (Malvern PSC100SM). The light source was a 50 mW argon ion laser (Ion-Laser-Technology, ILT 5000) operating at a wavelength of 488 nm. The time-dependent correlation function of the scattered intensity was obtained by using a 128-channel digital correlator (Malvern 7032 Multi-8 correlator), at 25 °C in a refractive index-matching liquid (toluene). The protein samples in HEPES buffers were filtered five times through a 0.22 µm filter (Millipore). For all the solutions in this study the intensity correlation data exhibited two distinct decay modes. Therefore we processed the data by using a method of least mean squares to fit the data to the sum of two exponential decays as follows. $\langle I(t)I(0) \rangle = A(1 + B_g(1)^2(t))$ (equation 1); $g(1)(t) = E_t \exp(-\Gamma_t t) + E_s \exp(-\Gamma_s t)$ (equation 2) where $I(t)$, $g(1)^2(t)$ and A are the scattered light intensity at time t , a time correlation function of the electric field of the scattered light and a constant, respectively. B in equation 1 is a constant depending on the coherence of the scattered light. E_t , E_s , Γ_t , Γ_s in equation 2 are constants determined by the method of least mean squares fit. If the light is scattered by Brownian particles, Γ_t and Γ_s are related to the diffusion constants of particles, D_t and D_s , as follows. $\Gamma_t = q^2 D_t$ and $\Gamma_s = q^2 D_s$, where $q = 4\pi n/\lambda_0 \sin(\theta/2)$ where n and θ are the refractive index of the solution and the scattering angle. The hydrodynamic radius of Brownian particles, R_h , can be evaluated using the Einstein-Stokes relation $R_h = kT/(6\pi\eta D)$ where η is the solvent viscosity.

Scanning electron microscopy. Ten µl of purified HPV-16 or -6b E7 protein (0.2 mg/ml) was fixed with 2% osmic acid anhydride and, after critical point dehydration, the material was coated with 20 Å of platinum, and observed by scanning electron microscopy with a JEOL JEL-2000 microscope.

SDS-PAGE and Western blots. Purified HPV-16 and -6b E7 proteins (10 µl, 0.1 mg/ml) were run on SDS-polyacrylamide gels using the Laemmli method and stained with silver or transferred to a nitrocellulose membrane filter for Western blotting. E7 protein bands were detected with hyperimmune mouse serum against purified HPV-16 E7 protein (polyclonal antibody), or with a monoclonal antibody against HPV-16 E7 protein, 8F (Tindle *et al.*, 1990), and visualized with the ECL system (Amersham).

pRB binding assay by immunoprecipitation. Purified E7 proteins [type 16, 16 mutant (24C-G) or 6b; each 20 µl, 0.25 mg/ml] were mixed with glutathione *S*-transferase (GST)-pRB bound to glutathione-Sepharose, washed three times with NETN buffer (Kaelin *et al.*, 1992) and the precipitated fraction was separated by 12.5% SDS-PAGE. After transfer to a nitrocellulose membrane, E7 protein bands were detected with the polyclonal antibody as described above.

[³H]TdR uptake in C127 cells. HPV-16, -16 mutant (24C-G) and -6b E7 proteins (each 0.5 ml, 200 µg/ml) were introduced into C127 cells (1×10^5 cells) at mid-log phase by osmotic shock (Okada & Rechsteiner, 1982). The cells were cultured in Dulbecco's MEM containing 10% fetal calf serum in the presence of 1 mCi [³H]TdR and the incorporation of radioactivity was measured during 4 h after introduction of the proteins. Triplicate samples were used for each E7 protein concentration.

Results

Crude extracts from *E. coli* cells were fractionated by ion-exchange chromatography on Q-Sepharose. HPV E7 proteins eluting at 0.5 to 0.6 M-NaCl were collected. The fractions with purities of more than 90% were concentrated from 30 ml to 5 ml by ultrafiltration and were subjected to gel filtration. The gel filtration patterns for both proteins are shown in Fig. 1. Three peaks for each protein are seen in the chromatograms. The first, second and third peaks appear to correspond to oligomers, dimers and monomers according to their molecular exclusion volumes, although the third peak of HPV-6b is very small. Selected fractions of the analysis shown in Fig. 1(b) were electrophoresed, transferred to nitrocellulose and probed with a monoclonal antibody against

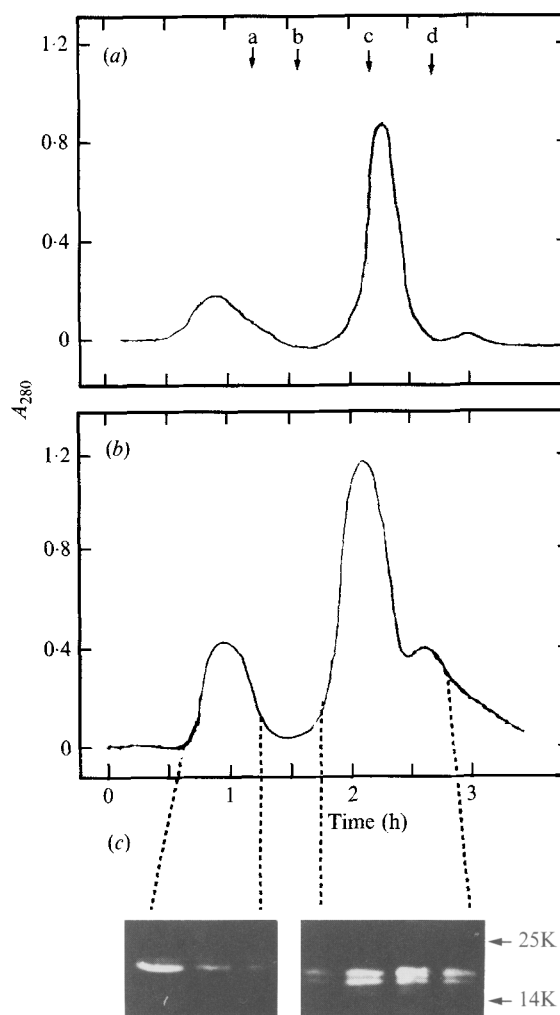


Fig. 1. Superdex-G75 elution patterns of HPV-6b (a) and -16 (b) E7 proteins. E7 protein fractions from ion-exchange chromatography were concentrated fivefold and applied to Superdex-G75. Fractions in (b) were Western-blotted with a monoclonal antibody against HPV-16 E7 protein (c). Markers were a, Albumin (67K); b, ovalbumin (43K); c, chymotrypsinogen A (25K); d, ribonuclease (13.7K).

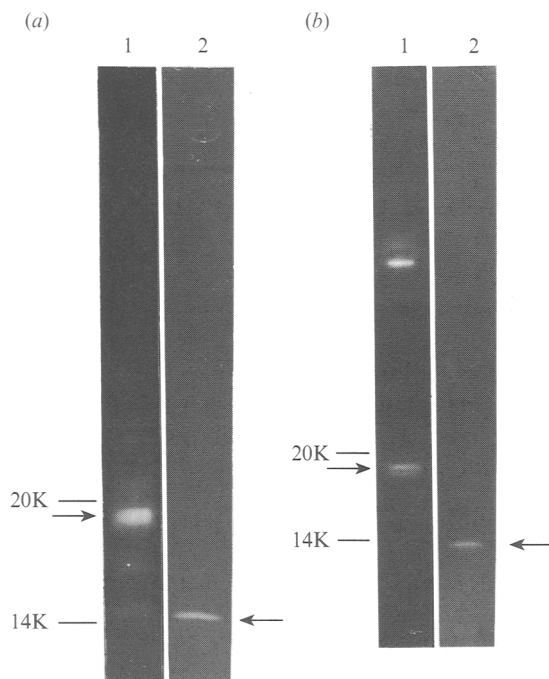


Fig. 2. SDS-PAGE (a) and Western blot (b) patterns of HPV-16 (lanes 1) and -6b (lanes 2) E7 proteins. The E7 proteins were purified as described in Methods, analysed by 12.5% SDS-PAGE, and stained with silver (a). The Western blot was performed as in Methods and E7 proteins were detected by the ELISA-ECL system (b). E7 bands are indicated by arrows.

HPV-16 E7 protein as described in Methods and are shown in Fig. 1(c). The material in the second peak with a molecular exclusion volume corresponding to chymotrypsinogen A was used in the following experiments as purified E7 protein. Purified HPV-16 and -6b E7 proteins were studied by SDS-PAGE and Western blotting as shown in Fig. 2. Their apparent mobilities at 19K and 14K are consistent with other reports (Gage *et al.*, 1990). The Western blots demonstrate that the polyclonal antibody used cross-reacted with HPV-6b E7 protein as well as with HPV-16 E7 protein.

To investigate the effect of protein concentration on oligomerization of E7 proteins, laser DLS was performed

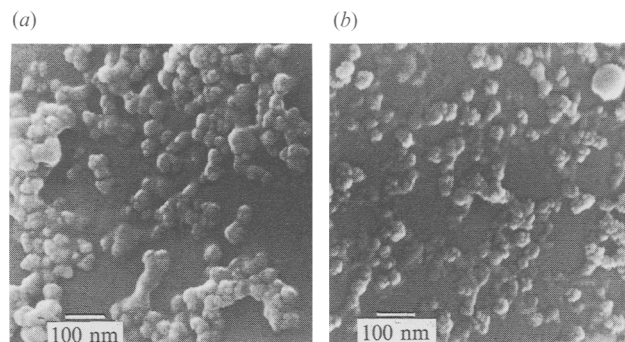


Fig. 3. Scanning electron microscopic observation of HPV-16 (a) and -6b (b) E7 proteins. Purified HPV-16 and -6b E7 proteins fixed as described in the text were coated with 20 Å of platinum and observed by scanning electron microscopy.

for various concentrations of protein solution. The hydrodynamic radii and diffusion constants were calculated and are shown in Table 1. Small-sized particles (the fast decay components R_f^H) increased in radius with increasing protein concentration whereas large-sized particles (slow decay R_s^H) did not. To visualize the protein particles, scanning electron microscopic examination of purified and concentrated HPV-16 and -6b E7 proteins was performed. Clustered protein complexes of approximately 20 to 30 nm diameter were seen for E7 proteins of both HPV-16 and -6b (Fig. 3). These features are consistent with the data from the light scattering.

To investigate whether the purified proteins had biological activities, pRB binding and the effect on DNA synthesis of purified E7 proteins were assessed as described in Methods. According to the results of coprecipitation experiments with GST-linked pRB(379-928), HPV-16 E7 protein produced in *E. coli* apparently retained its capacity to bind to pRB, whereas E7 of HPV-6b and HPV-16 (24C-G) mutant E7 bound less well (Fig. 4). The effects on [3 H]TdR incorporation as a measure of DNA synthesis of directly introducing the various proteins were examined and the results are shown in Fig. 5. Percentage differences in incorporation shown are relative to a control with the same amount of BSA. The

Table 1. Parameters of HPV-16 and -6b E7 proteins as measured by laser light scattering

HPV type	Concentration ($\mu\text{g/ml}$)	D_f^* ($\times 10^8$ cm^2/sec)	R_f^H (nm)	D_s^* ($\times 10^8$ cm^2/sec)	R_s^H (nm)
16	5	530 ± 255	1.45 ± 0.26	0.72 ± 0.37	240 ± 180
	30	127 ± 12.5	2.84 ± 1.35	2.02 ± 0.14	125 ± 18
	60	8.53 ± 0.23	28.8 ± 0.81	0.49 ± 0.11	205 ± 28
6b	30	104 ± 31.4	2.41 ± 0.70	0.85 ± 0.14	285 ± 40
	60	91.8 ± 0.08	26.9 ± 2.75	1.04 ± 0.29	216 ± 55

* D_f and D_s , Diffusion constant of fast and slow components of the decay curve.

† R_f^H and R_s^H are radii for fast and slow components, calculated as described in Methods.

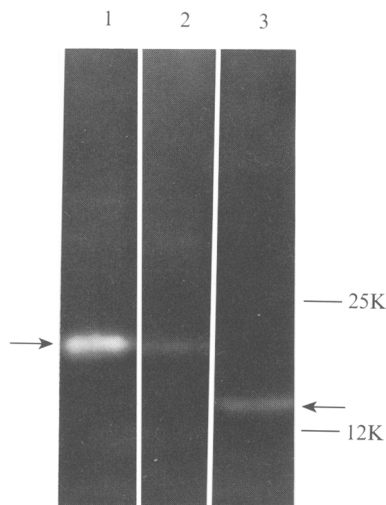


Fig. 4

Fig. 4. Retinoblastoma gene product binding assay by Western blotting for HPV-16 (lane 1), -16 mutant (24C-G) (lane 2) and -6b (lane 3) E7 proteins. Equal amounts of purified E7 proteins were mixed with glutathione–Sepharose-bound GST-RB(379-928) and coprecipitated E7 proteins were electrophoresed, transferred to nitrocellulose and detected with a polyclonal antibody.

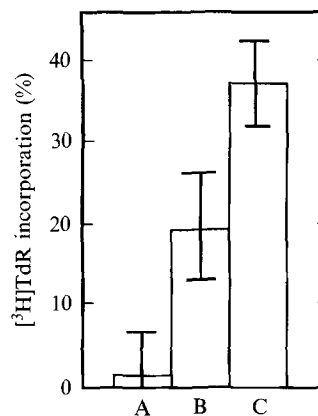


Fig. 5

Fig. 5. Effect of HPV E7 proteins on [³H]TdR incorporation in C127 cells. Purified E7 proteins (200 µg/ml) or BSA (200 µg/ml) were introduced into cells by osmotic shock with 1 mCi [³H]TdR, and 4 h later incorporated radioactivity was counted. The increases in incorporation as a percentage of the incorporation seen with BSA are plotted with standard error bars. A, HPV-16 mutant (24C-G) E7 protein; B, HPV-6b E7 protein; C, HPV-16 E7 protein.

HPV-16 E7 protein stimulated the incorporation of [³H]TdR when introduced into these cells. HPV-6b E7 protein showed a lower, but significant stimulation, whereas the HPV-16 (24C-G) mutant gave no significant increase in incorporation.

Discussion

In this study we showed by gel filtration, laser light scattering and scanning electron microscopy that HPV types 16 and 6b E7 proteins expressed in *E. coli* easily form oligomers. According to the results of gel filtration, when the protein concentration was 20 to 30 µg/ml, the most predominant form appeared to be the dimer. Next came large oligomers and only a small fraction was in the form of monomers. This distribution coincided with the results of light scattering experiments (Table 1), although monomers cannot be seen there. The Stokes' radius of E7 protein monomers considered as globular proteins was estimated from the M_r (11K) to be about 1.5 nm. By laser light scattering, monomer particles could be obtained for HPV-16 E7 protein at a concentration of 5 µg/ml. However, unfortunately, the ratio of monomers to oligomers cannot be determined by light scattering because diffraction from large oligomers is unusually enhanced. Oligomerization depended on protein concentration and at all the concentrations used in the experiments the level of oligomer formation, as judged

by scanning electron microscopy, was higher than the level as determined by light scattering. ¹H-NMR spectra of both types of E7 protein (at a concentration of 1 mg/ml) confirmed these results, showing similar broadening patterns, which demonstrates oligomer formation (data not shown).

What is the mechanism of oligomerization? Covalent bonding is one possibility because Patric *et al.* (1992) showed by X-ray absorption fine structure analysis that the HPV-16 E7 protein has no S–S bridges and two of the seven cysteine residues were exposed on the surface. However, when we concentrated E7 protein in the presence of 1 mM-DTT oligomerization still occurred. Furthermore, it was shown by monitoring with light scattering that addition of DTT did not destroy oligomers (M. Chinami, S. Sasaki & M. Shingu, unpublished). These results suggest that covalent bonding by intermolecular S–S bridges are not responsible for the oligomerization.

HPV-16 and -6b E7 proteins both possess zinc finger motifs at their carboxy termini and all the protein samples used in this study contained 20 µM-zinc acetate. Thus it is possible that multimerization occurred through the zinc finger motifs by way of bound zinc ions as McIntyre *et al.* (1993) hypothesized. We observed zinc ion-induced structural changes due to oligomerization of lacZ–HPV-16 E7 fusion proteins when analysed by ¹H-NMR (Chinami *et al.*, 1991). Dimerization through

metal linking of the Tat protein of human immunodeficiency virus (Frankel *et al.*, 1988) and of growth hormone (Cunningham *et al.*, 1991) have been reported.

The question remains whether oligomerization occurs only when the proteins are concentrated during the experimental procedures. We have shown that the oligomers of HPV-16 and -6b E7 proteins have the biological activities of binding pRB and stimulating DNA synthesis. The motif Cys-x-x-Cys in HPV-16 E7 protein is required for dissociation of E2F from pRB, the pRB binding sequence alone being insufficient for this dissociation (Huang *et al.*, 1993). In addition, Smotkin & Wettstein (1987) suggested that E7 protein from cervical cancer cells can form oligomers. Diffuse staining patterns of HPV-16 E7 proteins were often observed by immunofluorescence microscopy in cultured cells transfected with HPV-16 DNA (Sato *et al.*, 1989). Thus, the E7 protein oligomers may be functional and common to several HPV types, because the E7 proteins have a high degree of identity at the amino acid level and their chemical properties appear to be similar.

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