Characterization of Papillomavirus Polypeptides from Bovine Cutaneous Fibropapillomas

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SUMMARY

Virions of bovine papilloma virus (BPV) were isolated from a pool of cutaneous bovine fibropapillomas and purified by CsCl gradient centrifugation. SDS-PAGE revealed several polypeptides with an $M_r$ ranging from 76K to 19K. Western blot analysis of the viral isolate identified additional polypeptides when a rabbit anti-BPV serum was used, but only the main capsid component of 57K when a rabbit antiserum raised against human papillomavirus was used. The viral preparation was then $^{125}$I-labelled and further purified by gel filtration. SDS-PAGE of immunoprecipitates of the anti-BPV serum with different fractions from the chromatographic column revealed the polypeptides of 76K, 57K and 28K to be viral structural components. The 28K polypeptide, not previously characterized, was shown to be composed of several molecular forms, migrating over a pH range of 3.5 to 4.6 when analysed by two-dimensional PAGE. Following SDS-PAGE performed under non-reducing conditions, the 28K and 76K polypeptides and the main capsid component of 57K appeared to be linked by disulphide bridges to form hetero- or homopolymers.

The particles of papillomavirus isolated from infected tissues of different mammalian species show similar polypeptide compositions. Each type of virus contains a protein with an $M_r$ of 50K to 60K, assumed to be the main component of the viral capsid, a minor 76K peptide, four histone-like proteins and several variably detectable components of various $M_r$ values, some of which are likely to be degradation and/or cell-coded products (Favre et al., 1975, 1977; Gissmann et al., 1977; Orth et al., 1977, 1978; Pfister et al., 1977, 1979; Meinke & Meinke, 1981).

By immunoelectron microscopy (Gorra et al., 1985), radioimmunoprecipitation (Orth et al., 1978; Jenison et al., 1980) and immunoblotting (Roseto et al., 1984; Nakai et al., 1986; Komly et al., 1986), group-specific and type-specific determinants have been detected on the 50K to 60K polypeptide and on the 50K to 60K and 76K polypeptides, respectively. To characterize further the papillomavirus structural components, we analysed some biochemical and immunological properties of the proteins from bovine papillomaviruses (BPV). Purified viral preparations were obtained from a pool of bovine cutaneous fibropapillomas and consisted of clustered viral particles with full capsids, although numerous free capsomeres and a few particles with empty capsids were also observed (not shown). SDS–PAGE of the viral constituents on Coomassie Brilliant Blue-stained gels revealed two polypeptides of 76K and 57K and five polypeptides of lower $M_r$, three ranging from 37K to 33K (a doublet at 33K), one of 28K, and the smallest of 19K (Fig. 1, lane 1).

To distinguish between viral and non-viral polypeptides, the electrophoretically resolved viral preparation was analysed by Western blotting and the gels were developed with rabbit immune
Fig. 1. SDS-PAGE and Western blotting of purified BPV virions isolated from a pool of cutaneous bovine fibropapillomas. Keratinized epithelial layers were scraped from the lesions, homogenized and subjected to differential centrifugations including CsCl gradient equilibrium ultracentrifugation (Lancaster et al., 1976). The viral preparation was dissociated by incubation for 3 min in boiling water in the presence of 2% SDS and 5% 2-mercaptoethanol. Samples (10 µg) of dissociated viral proteins were run on 10% polyacrylamide slab gels with standard proteins to determine the Mr values. The gels were stained by a 0.25% solution of Coomassie Brilliant Blue and destained with 7% acetic acid and 5% methanol (lane 1). The viral polypeptides were transferred electrophoretically to a nitrocellulose membrane by Western blotting. After saturation with 3% bovine serum albumin (BSA) blots were incubated for 2 h at 20 °C with a rabbit antiserum (1/200 dilution) against SDS-disrupted BPV virions (Dako, Santa Barbara, Ca., U.S.A.) (lane 2) or a rabbit antiserum (1/200 dilution) against SDS-disrupted HPV virions (a gift from Dr K. V. Shah of the Johns Hopkins University School of Hygiene and Public Health, Baltimore, Md., U.S.A.) (lane 3). As a control, NRS was used at the same dilution (lane 4). After washing, 125I-labelled goat anti-rabbit IgG (2 × 10⁶ c.p.m./ml) was added. The immunologically recognized polypeptides were visualized by autoradiography.

Sera against BPV or human papillomavirus (HPV) disrupted virions. Lane 2 of Fig. 1 shows that the anti-BPV serum identified the 57K polypeptide and several species of different Mr in particular, a 46K molecule, not detected in the stained gel, was strongly reactive and no evidence of the 76K component was found. The anti-HPV serum identified only the 57K component (Fig. 1, lane 3). The specificity of the immunoblots was demonstrated by the lack of reactivity of normal rabbit serum (NRS) (Fig. 1, lane 4). The reactivity of the anti-HPV serum being restricted to the 57K protein suggested that some of the unidentified polypeptides recognized by the anti-BPV serum could have been specific BPV components and other cellular contaminants of the viral preparation originally used to prepare the immune serum, as suggested by other studies (Favre et al., 1975; Meinke & Meinke, 1981). We therefore attempted to purify the viral preparation further.

The viral preparation was iodinated with 125I under non-dissociating conditions and analysed by SDS–PAGE in the presence of 2-mercaptoethanol. The autoradiogram in Fig. 2 (b) (lane A) shows bands that correspond in number and migration properties to those detected on the stained gel of Fig. 1 (lane 1). The absence of the 19K component in Fig. 2 (b) (lane A) is due to different migration conditions, as shown in other experiments with the labelled viral preparation (see Fig. 2c, lanes A and B). The iodinated viral preparation was then gel-filtered under non-dissociating conditions on a Sephadex G-150 column to recover intact virions in the void volume and to separate free proteins. Fig. 2(a) shows the elution profile of the radioactivity recovered from the column and the fractions containing the calibration markers. Most of the fractions of the column were then analysed by SDS–PAGE, which enabled identification of three regions each containing the same polypeptides. The autoradiogram in Fig. 2(b) shows the results of an SDS–PAGE analysis of some fractions of the three regions. The void volume fractions contained 76K, 57K and 28K polypeptides (lanes 1). In additional experiments performed under different migration conditions, low Mr components were also detected (see Fig. 2c, lane B). In the fractions of the second region only 57K and 28K components were
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Fig. 2. Gel filtration chromatography and SDS-PAGE of radiolabelled virions. The viral preparation (5 µg) was iodinated with 1 mCi of carrier-free Na\textsuperscript{125}I (Amersham) and chloramine T (Hunter & Greenwood, 1962), and separated from free iodine by filtration on a Sephadex G-25 column (20 × 1 cm) equilibrated in 10 mM-Tris–HCl, 0.25% BSA and 150 mM-NaCl, pH 7.4. (a) The iodinated viral preparation was applied to a Sephadex G-150 column (30 × 1.5 cm) equilibrated in 10 mM-Tris–HCl containing 150 mM-NaCl, pH 7.4, and then eluted with the same buffer at a flow rate of 24 ml/h. The column had been previously calibrated with the standards blue dextran (BD), BSA and phenol red (PhR); their elution positions are indicated. Fractions of 0.6 ml were collected at 1.5 min intervals and counted in a gamma counter. (b) The fractions corresponding to the void volume (lane 1), or the 150K to 70K molecules (lane 2) or the 70K to 30K molecules (lane 3) were pooled and analysed by SDS-PAGE on 12.5% slab gels under reducing conditions. Gels were then fixed, dried and subjected to autoradiography. Lane A indicates the protein pattern of the iodinated viral preparation before chromatography. (c) A portion (200 µl) of each pool obtained from the different fractions of the Sephadex column and containing about 10\textsuperscript{5} TCA-precipitable c.p.m. was incubated overnight at 4°C with 5 µl of rabbit anti-BPV serum or NRS. Staphylococcus aureus Protein A (100 µl) (Pansorbin; Calbiochem) was added, and the mixtures were incubated for 2 h at 4°C. Precipitates were centrifuged, washed, and after dissociation in a boiling 2% SDS solution containing 5% 2-mercaptoethanol, were analysed by SDS–PAGE in 12.5% or 7.5% slab gels. The radioactive polypeptides were revealed by autoradiography. Pooled fractions of void volume (lane 1), region 2 (lane 2) and region 3 (lane 3). Polypeptide pattern of non-filtered viral preparation before (lane A) and after (lane B) immunoprecipitation with the same antiserum.

identified (Fig. 2c, lane 2), and the polypeptides of 33K to 37K were included in the third region (Fig. 2c, lane 3). The main conclusion was that the components of 33K to 37K were not included in the fractions containing the putative intact virions. The iodinated polypeptides, present in the viral preparation and in the pools of the fractions related to the three different regions obtained in the gel filtration experiment, were reacted with the anti-BPV serum, and the immunoprecipitates were analysed by SDS–PAGE under reducing conditions. Fig. 2(c) shows that the immune serum immunoprecipitated all the polypeptides except the 33K to 37K components from the initial viral preparation (compare lanes A and B). The same qualitative results were obtained with the pool of the fractions of the first region (lane 1), whereas from the fractions of the second region, which lacked the 76K component, only the 57K and 28K polypeptides were
Fig. 3. Two-dimensional PAGE of radiolabelled purified BPV proteins as described by O'Farrell (1975). Purified $^{125}$I-labelled BPV proteins immunoprecipitated by rabbit anti-BPV serum were isoelectrically focused in a 7.5% reducing slab gel with a pH gradient from 6.36 (left) to 3.41 (right) obtained with a mixture of Ampholines (LKB). The second dimension run was in a 12.5% SDS–PAGE slab gel under reducing conditions. The separated iodinated polypeptides were detected by autoradiography.

immunoprecipitated (lane 2). No reactivity was obtained with the 33K to 37K proteins of the third region of the gel filtration eluate (lane 3).

Thus, the viral preparation and the immunoprecipitates were found to contain the 76K and 57K proteins already reported as viral structural components, and a 28K polypeptide previously detected but not characterized (Pfister et al., 1979). To analyse this molecule further, the immunoprecipitate produced by using the intact virions contained in the void volume of the column (region 1) and the rabbit anti-BPV serum was resolved by two-dimensional gel electrophoresis. Fig. 3 shows that the individual polypeptides of 76K, 57K and 28K were composed of different molecules of the same $M_r$ but with variable charge, most likely due, as for polyoma viruses, to different degrees of phosphorylation (O'Farrell & Goodman, 1976; Ponder & Crawford, 1977). The 28K polypeptide displayed the greatest charge heterogeneity, since it was composed of molecules migrating in a pH range of 3-5 to 4-6. The distinct pattern of charge heterogeneity and the chromatographic behaviour of the 28K component render it unlikely that this polypeptide was a degradation product of the 76K or 57K peptides. This point was supported further by an experiment in which the products of the spontaneous endogenous proteolytic degradation of a labelled viral preparation were examined. No evidence for proteolytic generation of the 28K component was found (not shown). Two other distinct isolates of BPV were obtained from two pools of bovine skin fibropapillomas, and in both instances the molecule was promptly identified (data not shown).

Finally, a clear structural relationship between the 76K, 57K and 28K molecules was demonstrated in SDS–PAGE experiments performed under non-reducing conditions after immunoprecipitation of the viral polypeptides with the anti-BPV serum. As shown in Fig. 4(a), whereas the 76K component appeared unchanged, the 57K and 28K polypeptides were no longer detectable, and a band of about 200K was evident (compare lanes 1 and 2). Following the method described by Cleveland et al. (1977), the region of the gel containing the 200K component was excised and electrophoresed under reducing conditions in a second gel system. Most of the 200K molecules divided into the two components of 57K and 28K (lane 3).

To rule out the possibility that the chemical behaviour and stability of viral proteins may have been changed by the labelling procedure, the proteins of unlabelled virions were resolved by SDS–PAGE under reducing and non-reducing conditions, and the resulting blots obtained after Western blotting were directly stained or developed with anti-BPV serum. Fig. 4(b) lane 1 shows a blot performed under non-reducing conditions and stained directly with Ponceau red; the 200K band is evident. A parallel blot in lane 2 developed with anti-BPV serum revealed that the same 200K component was immunologically recognizable. The 200K band was excised from the
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Fig 4. SDS-PAGE and Western blotting of purified BPV virions under reducing or non-reducing conditions. (a) SDS-PAGE on a 10% slab gel under reducing (lane 1) or non-reducing (lane 2) conditions of [125I]-labelled viral proteins immunoprecipitated by a rabbit anti-BPV serum (see legend to Fig. 2). The 200K band cut from lane 2 was analysed by SDS-PAGE on a 12.5% slab gel under reducing conditions (lane 3). (b) Western blotting (see legend to Fig. 1) following SDS-PAGE of the viral preparation run under non-reducing conditions: staining with Ponceau red (lane 1); reaction with rabbit anti-BPV serum (lane 2). Western blotting following SDS-PAGE of the isolated 200K component run under reducing conditions: staining with Ponceau red (lane 3); reaction with rabbit anti-BPV serum (lane 4).

gel and run under reducing conditions. Following the blotting, the staining of the nitrocellulose with Ponceau red (lane 3) indicated that most of the 200K molecules were resolved into the 76K, 57K and 28K proteins. After the addition of anti-BPV serum to the blot (lane 4), only the 57K protein was recognized, suggesting that neither the 76K nor the 28K viral component expressed antigenic epitopes reacting with the rabbit anti-BPV serum.

The latter experiments indicate that, in their native form, the three peptides are linked by disulphide bridges to form homo- or heteropolymers of high Mr.

Since only the 57K component was detected reproducibly by Western blotting and immunoprecipitation, the 76K and 28K components are likely to be indirectly immunoprecipitated when still associated with the immunosensitive 57K molecule.

Although it has been established that the 57K peptide is encoded by the L1 open reading frame (Danos et al., 1984; Pilacinski et al., 1984) and that the 76K protein is encoded, at least partially, by the L2 open reading frame (Komly et al., 1986), the origin of the 28K component is still obscure. Further studies will analyse the possibility that this molecule might originate from the splicing of mRNA covering the whole L region or from post-translational modifications of the 76K component itself.

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