Calcium is required in reassembly of bovine papillomavirus \textit{in vitro}

Jeanette Paintsil,\textsuperscript{1} Martin Müller,\textsuperscript{1} Maria Picken,\textsuperscript{2} Lutz Gissmann\textsuperscript{1}\textdagger\textsuperscript{1} and Jian Zhou\textsuperscript{1}

Departments of Microbiology & Immunology\textsuperscript{1}, and Pathology\textsuperscript{2}, Loyola University Medical Center, 2160 S. First Ave, Maywood, IL 60153, USA

Papillomaviruses are small DNA viruses which infect and induce benign warts and sometimes malignant tumours in the epithelium of the skin or mucosa. The viruses do not replicate in conventional tissue culture systems and little is known about the requirements for virus assembly. We investigated the effect of ethylene glycol-bis(aminoethyl ether)-tetraacetic acid (EGTA) and dithiothreitol (DTT) treatment on the stability of bovine papillomavirus type 1 (BPV-1) particles \textit{in vitro}. Removal of calcium ions by 11 mM EGTA at pH 8.0 together with reduction of disulfide bonds by 15 mM DTT destabilized BPV particles. Electron microscopy examination of treated particles showed that the BPV particles had been disrupted to capsomeres. Addition of exogenous calcium ions to the disruption buffer prevented virus destabilization. Adding calcium to the disrupted BPV particles resulted in the reassembly of disrupted particles. The reassembled particles were morphologically similar to intact BPV virions. We further quantified the efficiency of reassembly by focus formation assay. We recorded 500-fold less infectivity for reassembled BPV and 4-fold less haemagglutination activity compared to untreated BPV, pointing towards a decrease in the amount of reassembled particles recovered.

Introduction

Calcium is required in the assembly process of capsids of many different viruses including simian virus 40 (SV40) and mouse polyomavirus (Montenarh & Henning, 1983; Brady \textit{et al.}, 1977), and may play a role in virus stability (Durham \textit{et al.}, 1977; Durham & Haidar, 1977; Durham & Hendry, 1977; Fox \textit{et al.}, 1997; Drzenick & Bilello, 1972; Shirley \textit{et al.}, 1981; Zhao \textit{et al.}, 1997; Michelangeli \textit{et al.}, 1995). The presence of calcium or other cation(s) in papillomavirus virions has not yet been reported.

The papillomaviruses are a heterogeneous group of epitheliotropic DNA viruses (zur Hausen, 1991; Zachow \textit{et al.}, 1982). The properties of papillomaviruses include small size, double-stranded circular DNA genome of about 8 kb and a nonenveloped icosahedral capsid (Crawford & Crawford, 1963; Galloway & McDougall, 1989). This virus shell has a diameter of about 55 nm and is composed of the major capsid L1 protein (55 kDa) and the minor capsid L2 protein (50 kDa) (Doorbar & Gallimore, 1987; Komly \textit{et al.}, 1986).

The L1 protein forms virus-like particles (VLPs) when expressed alone in recombinant expression systems (Zhou \textit{et al.}, 1993; Kirnbauer \textit{et al.}, 1992, 1993; Hagensee \textit{et al.}, 1993; Rose \textit{et al.}, 1993). However, a detailed study of papillomavirus capsid assembly is elusive, as there is no suitable cell culture system for the efficient propagation of papillomavirus particles. Understanding the details of virus assembly may improve the design of antiviral strategies and be important for therapy. \textit{In vitro} assembly studies have shown that mouse polyomavirus capsids can be disrupted in the presence of chelating and reducing agents (Brady \textit{et al.}, 1977, 1978). This disruption is a result of calcium removal from the capsid, thus destabilizing the virus. Reassembly of the disrupted capsid after addition of exogenous calcium has been reported (Brady \textit{et al.}, 1979; Yuen & Consigli, 1982).

Using the high affinity of ethylene glycol-bis(aminoethyl ether)tetraacetic acid (EGTA) to chelate calcium ions, in addition to disulfide bond disruption by dithiothreitol (DTT),
we show that bovine papillomavirus (BPV) particles can be disrupted and subsequently reassembled in vitro.

Methods

■ Virus purification and analysis. BPV particles were purified from bovine warts as previously described (Zhou et al., 1995). Supernatant from homogenized wart tissue was layered on top of a 40% sucrose cushion, followed by centrifugation in a Sorvall AH629 rotor at 27000 r.p.m. for 2 h. The resulting pellet was purified by CsCl (1.34 g/ml) centrifugation at 45000 r.p.m. for 18 h in a Beckman 70 Ti rotor. Aliquots of gradient fractions were separated by SDS–PAGE followed by Coomassie blue staining and Western blot analysis. To identify the purified papillomavirus, 50 μl of the virus sample was disrupted in 1% SDS for 1 h at 37 °C followed by proteinase K digestion (50 μg/ml). The viral DNA was extracted by the phenol–chloroform method and precipitated with ethanol. purified BPV DNA was cleaved with restriction enzymes (BamHI, AccI, DraI, BstI, PslI). After agarose gel electrophoresis the DNA fragments were compared to corresponding fragments of cloned BPV-1 genomic DNA.

■ Disruption assay. Purified BPV-1 particles were dialysed against 1 mM HEPES (pH 7.4) at room temperature for 1 h on microfilters (Millipore, type VS 0.25 μm) and dialuted 1:1 with PBS (pH 7.2). A 20 μl sample of the dialysed virus was transferred onto poly-l-lysine-treated (1 mg/ml) carbon-coated copper grids, stained with 2% uranyl acetate and examined with a Zeiss EM 900 electron microscope (EM) at 80 kV. Virus particles were disrupted by adding dialysed virions to an equal volume of disruption buffer (100 mM Tris–HCl, pH 8.0, 22 mM EGTA, 150 mM NaCl, 30 mM DTT) at room temperature for 3 h (Brady et al., 1977, 1978).

■ Virus reassembly. For virus reassembly, a solution of disrupted virus was divided into two parts. One part was used for EM examination. An equal volume of a ‘stop’ solution (25 mM CaCl₂, 20% DMSO) was added to the second part of the sample and incubated at room temperature for 30 min. This sample was then dialysed overnight at 4 °C against reassembly buffer (150 mM NaCl, 25 mM CaCl₂, 10% DMSO, 0.01% Triton X-100, 100 mM Tris–acetate, pH 5.0) (Brady et al., 1979; Yuen & Consigli, 1982). Reassembly was examined by EM.

■ DNase I treatment of BPV samples. The disrupted BPV sample was divided into four parts. One part was set aside for a control and another part used for reassembly. The remaining two parts were digested with DNase I (30 units; Boehringer Mannheim) at room temperature for 1 h. One half of the DNase-treated sample was then reassembled. After EM examination, all the samples were then used to infect C127 cells for focus formation. DNase digested untreated and reassembled BPV virions served as controls.

■ Disassembly inhibition assay. The effect of exogenous cations on virus disruption was determined by adding excess CaCl₂ or MgCl₂ (10 mM in each case) to the disruption buffer 30 min prior to addition of BPV particles. The effect of antibodies specific for L1 and L2 capsid proteins on virus stability was also determined. Rabbit anti-papillomavirus antibodies (DAKO) or rabbit anti-BPV L2 full-length antibodies (kindly provided by J. Schiller, NIH, Bethesda, MD, USA), both at a 1:50 dilution in PBS, were pre-incubated with BPV particles for 30 min at room temperature before adding disruption buffer. Virus disruption was then carried out as described above and monitored by EM.

■ Immunogold labelling. Untreated and disrupted virus particles were transferred onto copper grids and incubated with various antibodies at a 1:10 dilution in blocking buffer (1% BSA in PBS) at room temperature for 1 h (Hagensee et al., 1993). Grids were then washed four times with blocking buffer for 1 min each followed by incubation with gold-labelled secondary antibodies at a 1:20 dilution in blocking buffer. After incubation for 1 h, grids were washed four times in water and stained for 5 min with 2% uranyl acetate, followed by EM.

■ Haemagglutination assay (HA). Twofold serial dilutions (1:10–1:2560) of BPV samples starting from 50 ng were made in PBS. An equal volume of 1% mouse erythrocytes was added, mixed and incubated at 4 °C for 3 h. Plates were photographed and HA titre was recorded as previously described (Favre et al., 1974; Roden et al., 1995, 1996).

■ Cell culture and focus assay. C127 cells were grown in DMEM supplemented with 10% FCS at 37 °C in a humidified atmosphere (5% CO₂). For the focus assay, 2.5 × 10⁴ cells in DMEM–5% FCS were seeded into 12-well plates and inoculated with comparable dilutions of either untreated, disrupted or reassembled BPV virions. Mock-treated cultures were used as controls. Four hours post-infection, the inoculum was removed and fresh medium was added. The medium was replaced the next day with DMEM–2% FCS, followed by changes every 3 days for 21 days. Finally, cells were rinsed twice with PBS, fixed in ice-cold methanol for 3 min followed by 15 min staining in 0.5% methylene blue–0.25% basic fuchsin in methanol (Dvoretzky et al., 1980; Roden et al., 1994); foci were then counted.

■ Southern blot analysis. Individual foci from C127 cultures inoculated with untreated, disrupted and reassembled BPV as well as from control cells were picked and cells expanded in 6-well plates. After 3 weeks, cellular DNA was extracted. Cells were washed once in PBS and trypsinized. Serum-free DMEM (1 ml) was added and the cells were collected into 2 ml tubes. Cells were pelleted at 1000 r.p.m. for 10 min, washed once with 20 mM Tris–HCl (pH 8.0) and resuspended in 400 μl of the Tris–HCl buffer. Lysis buffer (200 μl) (3% SDS, 70 mM Tris–HCl, pH 8.5) containing proteinase K (60 μg/ml) was added. Samples were incubated at 52 °C for 2 h. DNA was then extracted by the phenol–chloroform technique and ethanol precipitated. The DNA was cleaved with BamHI, separated on 1% agarose gel, transferred onto nylon membrane and hybridized with radiolabelled BamHI-linearized BPV-1 genomic DNA. DNA was extracted from BPV wart tissue accordingly and used as a control.

Results

BPV-1 virions can be disrupted by EGTA and DTT in vitro

BPV-1 particles were extracted from bovine skin warts and purified by CsCl gradient centrifugation. BPV-1 virus particles used for the experiments are shown in Fig. 1(A). The purified virus particles were uniform in size with an estimated 50 nm diameter. Purified SV40 particles used as disruption control had a very similar structure to BPV but were smaller in size, about 45 nm (data not shown).

Incubation of BPV virions in 11 mM EGTA and 15 mM DTT resulted in their disassembly (Fig. 1B). Virus disruption was visualized by EM and monitored by haemagglutination. Disruption of virus particles was observed when both DTT
and EGTA were present in the buffer. Disruption was first noted 15 min after the treatment and was nearly complete within 60 min of incubation at room temperature. EM revealed many capsomeres in the preparation suggesting that the capsomere subunits were resisting further disruption (see arrows in the insert; Fig. 1B). Occasionally, partially disrupted particles were found after 60 min of treatment (Fig. 1B). Increasing the incubation time of the disruption experiments to 3 h led to complete disruption of the particles. This length of treatment was used throughout the following experiments, except for the particles used for immunogold labelling. However, BPV particles were not completely disassociated by treatment with DTT alone or EGTA alone. This result was surprising as human papillomavirus type 33 (HPV-33) VLPs could be completely disrupted by DTT treatment (Sapp et al., 1995). To test if the difference observed was due to the full BPV particle being more stable than the VLP, we compared the morphology of BPV particles and BPV VLPs treated with 15 mM DTT. It was found that the VLPs were completely disrupted by the reducing agent (Fig. 2C, D), while only a fraction of the BPV particles were destroyed by the same treatment (Fig. 2A, B). This suggested that the structural difference between full particles and VLPs resulted in the full BPV being resistant to DTT treatment. It is also possible that the stability of different types of papillomaviruses varies, as HPV-11 assembled from E. coli-expressed L1 is resistant to treatment with EGTA or DTT (Li et al., 1997). To determine the location of the viral DNA and capsid proteins after disruption it was necessary to use partially disrupted particles. Therefore a shorter incubation period of 30 min was used for this part of the study. We investigated the fate of the viral DNA after disruption. Immunogold labelling experiments using anti-L1/anti-DNA (not shown) and anti-L2/anti-DNA antibodies revealed the location of the viral DNA in relation to the disrupted particles (Fig. 1C) while irrelevant antibody did not bind to the BPV structure. Although the study was not...
quantitative, we frequently observed that the DNA had been released from partially disrupted particles (5 nm gold particles indicated by the bottom arrow). Additionally, L2 was closely associated with the particles as depicted in the micrograph (15 nm gold particles; see top arrow).

Reassembly of disrupted BPV particles

We found by EM that after overnight dialysis against an EGTA- and DTT-free calcium-supplemented reassembly buffer at pH 5.0, disrupted particles had reassembled. Tris–acetate buffer was used to lower the pH of the reassembly buffer to prevent EGTA from chelating the added calcium. No capsomers were visible in the preparation and the reassembled particles, about 50 nm in diameter, had a structure similar to untreated BPV virions (Fig. 1D). The efficiency of reassembly was further quantified by focus formation assay (see below). Disrupted virus particles did not reassemble into capsids when Ca\(^{2+}\) was omitted from the reassembly buffer, indicating that this cation was essential for assembly. Disrupted samples left at 4 °C for over a month could still be reassembled into full particles indicating that, given the right conditions (i.e. pH, Ca\(^{2+}\)), disrupted virus could be efficiently reassembled (data not shown). We tested the range of pH from 4.0 to 8.0 and found pH 5.0 to 6.5 to be ideal for reassembly.

Calcium and BPV-1 L2-specific antiserum stabilize BPV

We determined the effect of exogenous Ca\(^{2+}\) and Mg\(^{2+}\) on the stability of BPV virions and found that in the presence of Mg\(^{2+}\), the virus could still be disrupted by EGTA and DTT. Addition of Ca\(^{2+}\) prevented BPV disruption in the presence of EGTA/DTT, suggesting that Ca\(^{2+}\) stabilized the virus structure. We also investigated the effect of antibodies to capsid proteins on BPV disruption. We showed that the presence of antibodies to linear sequences of L1/L2 (Fig. 3A; DAKO anti-BPV) partially protected BPV against disruption by EGTA/DTT, as did rabbit anti-BPV L2 (Fig. 3B). This suggests that antibodies against L1 or L2 capsid proteins could stabilize the papillomavirus structure, although the anti-L2 antiserum seemed more effective in preventing BPV disruption (Fig. 3A, B). The mechanism by which anti-L2 antibody prevents particle reassembly is not clear as L1 alone could assemble into VLPs; we speculate that the L2–IgG complex could still interact with L1 protein, resulting in disruption of L1 assembly. The antibodies to either L1 or L2 prevented complete reassembly of disrupted virus particles equally well (Fig. 3C, D respectively), indicating that both capsid proteins were required for BPV reassembly. Although there were a few particles reassembled from disrupted BPV virions in the presence of antibodies, most of them were empty virus particles.
We then performed haemagglutination assays (HA) for BPV particles that had been treated with EGTA/DTT in the presence of antibodies to L1 or L2 (Fig. 4). None of the antisera used inhibited HA by BPV as has been previously reported (Roden et al., 1995). An HA titre of 160 was recorded for the particles disrupted in the presence of polyclonal serum against BPV particles (non-neutralizing antibody to linear epitopes of L1) or neutralizing rabbit antiserum against L2 protein made in E. coli. (neutralizing antibody; Liu et al., 1997). This represents a 4-fold decrease in haemagglutination activity compared to the untreated full BPV particles, which had a titre of 640. Treatment with MAb 5B6, which recognizes a conformational epitope (anti-L1; Roden et al., 1995) and anti-L2 MAbs C1, C3 and C6, which recognize L2 on the surface of BPV-1 particles (Liu et al., 1997), also prevented disruption of BPV, resulting in comparable HA titres of 160. The results for MAbs C1 and C3 are not shown. Disrupted BPV particles did not show any activity (at a concentration of 1:10), indicating that BPV capsomers are unable to haemagglutinate mouse erythrocytes. It is not clear if the DTT present in the disruption buffer had an effect on the integrity of antibodies used in these assays. However, the stabilization effect may be derived from the direct contact between the antibody and the particles.

**Infectivity of reassembled BPV**

In order to determine the biological activity of the reassembled virus we utilized the BPV transformation assay on C127 cells (Lowy et al., 1980; Dvoretzky et al., 1980). The results are averages from 20 independent experiments each performed in duplicate. We recorded a significant 10-fold increase in focus formation by reassembled over disrupted virus ($10^4$ vs $10^3$ foci; Student’s $t$-test $= 6.64; P \leq 0.01$) after subtracting foci numbers from uninfected controls. The titre of reassembled BPV was about 500-fold lower than untreated BPV ($5 \pm 5 \times 10^4$). C127 cells spontaneously form foci in the absence of BPV infection. We therefore determined the number of foci that contained BPV DNA after infection with untreated, disrupted and reassembled particles. Southern blot analysis of DNA extracted from 18 individual foci from triplicate experiments for each treatment showed that $5 \pm 5(18)$ foci from reassembled virus and $12 \pm 8(18)$ foci from untreated virus contained BPV DNA. Only one focus (1/18) was BPV positive from the disrupted virus. None of the 18 foci from the controls was positive for BPV DNA.

In order to test whether DNA in the reassembled virions was completely packaged, we performed a DNase digestion of...
disrupted and reassembled BPV particles prior to the focus assay (Fig. 5). Focus formation by untreated virions is shown in Fig. 5(A). Background foci were noted in DNase buffer or mock-infected cell controls, likewise for disrupted particles (Fig. 5B), disrupted particles treated with DNase (Fig. 5C) and empty particles assembled after DNase treatment (Fig. 5D). This suggests that the viral DNA was released. DNase treatment after reassembly did not significantly reduce the number of foci (3 × 10⁴), indicating that viral DNA was protected after reassembly (E and F, respectively). We determined the efficiency of the digestion by using equivalent amount of DNase on 5 µg of 8 kb BPV DNA. Complete digestion was verified by agarose gel electrophoresis and Southern blot analysis (data not shown), indicating that 30 units of DNase completely removed the BPV genome from disrupted particles. Treatment of BPV particles with DTT or EGTA alone reduced the number of foci by 500-fold, producing 1 × 10⁴ foci (data not shown). Because disruption of BPV by DTT/EGTA treatment produced 10⁴ foci, it followed that using only one of DTT and EGTA resulted in only partial disruption of BPV particles, which was also confirmed by EM.

Using the same disruption and reassembly conditions we could disrupt and subsequently reassemble BPV L1 VLPs, suggesting that calcium plays a role in papillomavirus capsid assembly. Reassembled VLPs had the characteristic virion structure, about 50 nm in diameter and an electron-dense centre, indicating the absence of DNA. After disruption only capsomere subunits were detectable by EM. After reassembly no capsomere was found. Since BPV L1 VLPs are devoid of
DNA, we did not determine biological activity of the reassembled VLPs. Instead, we performed HA analysis. Reassembled VLPs, unlike disrupted VLPs, haemagglutinated mouse erythrocytes (Fig. 6). Therefore, capsomeres do not seem to have HA activity at a 1:5 dilution of disrupted particles (0.1 µg in PBS). The HA titre for reassembled VLPs was 1:20 and that for untreated VLPs was 1:80. The presence of the disruption and reassembly buffers had no effect on the HA activity of BPV-1 particles. We observed no difference in the results when the samples were dialysed before use in HA and focus formation assays.

Discussion

A number of studies have demonstrated that calcium is required for maintaining the stability of viruses such as mouse polyomavirus and SV40, and forms an integral part of the virus (Yuen & Consigli, 1982; Montenarh & Henning, 1983). We used the mouse polyomavirus disruption assay described by Brady et al. (1977) to determine whether papillomaviruses could be disrupted and subsequently reassembled in vitro. In this study, we present evidence that BPV particles can be disrupted by EGTA and DTT. These disrupted particles were unable to haemagglutinate mouse red blood cells and were not infectious. Exogenous calcium specifically inhibited this disruption suggesting a possible role for calcium in papillomavirus stability. The complete disruption of BPV virions requires both DTT and EGTA. While DTT alone could disrupt HPV VLPs (Sapp et al., 1995) full BPV virions are more stable than VLPs. Therefore, the disassembly conditions described by Sapp et al. for VLPs cannot be applied to similar studies on infectious papillomaviruses.

The reassembly of disrupted virus particles on addition of calcium has also been reported for SV40 and mouse polyomavirus (Montenarh & Henning, 1983; Brady et al., 1979; Yuen & Consigli, 1982). Our results show that disrupted BPV particles can be reassembled into full particles when calcium in molar excess of EGTA is used. EM showed that the reassembled virus particles were similar in morphology to the untreated purified BPV particles. Moreover, these reassembled particles regained biological activity, namely transformation of C127 cells and haemagglutination of mouse erythrocytes.

In a biological assay, DNase treatment of reassembled BPV did not lead to a reduction in numbers of foci, indicating the presence of packaged and thus protected DNA. Viral DNA was recovered from foci infected with these reassembled virus particles which showed that they were infectious. BPV DNA was recovered from 1/18 foci obtained after infection of cells with disrupted particles, but the level of transformation from such disrupted BPV was similar to background level. The reassembly conditions for BPV still need to be optimized in order to achieve a higher efficiency of reassembly (10^4 reassembled versus 5.5 x 10^4 untreated). The results obtained in our study are comparable to the data from mouse polyomavirus disruption and reassembly studies. Brady et al. (1979) observed an 8-fold decrease in haemagglutination activity for the reassembled virus and a decrease in infectivity rate of about 7000-fold. We found a 4-fold decrease in haemagglutination activity and about 500-fold decrease in infectivity of reassembled BPV virions. Unlike polyomavirus, BPV capsomeres do not haemagglutinate mouse red blood cells.

Antisera to capsid proteins stabilized the virus structure in the presence of EGTA/DTT and after disruption also prevented complete reassembly. Stabilization by anti-L2 may be due to the binding of L2 antibody to two adjacent L2 molecules displayed as a loop on the surface of the virus (Liu et al., 1997). This could result in cross-linking of capsid subunits which may be linked by either disulfide bonds or calcium. If the position of L2 in papillomavirus particle is in the centre of vertex capsomeres as suggested by cryomicroscopy (Trus et al., 1997), the L2 antibody may not be able to cross-link the molecules due to the distance. It requires further study to determine the mechanism. Although the role of L2 in virus assembly is not clear, this observation suggests an important role for L2 in assembly of infectious BPV particles. It is known that the L2 protein binds the viral DNA and also enhances the efficiency of L1 protein assembly into VLPs (Zhou et al., 1994; Hagensee et al., 1993). From the immunogold labelling results, we assume that part of L2 is exposed on the surface (Liu et al., 1997) and is closely associated with the capsid upon disruption. Probably L2 is necessary for positioning the viral DNA into the capsid during assembly (Zhou et al., 1994).

Our results show clearly that disrupted BPV particles can be reassembled in vitro. We know from previous studies that recombinant L1 has the capacity to self-assemble into VLPs (Zhou et al., 1993; Kirmbauer et al., 1992, 1993; Hagensee et al., 1993; Rose et al., 1993). Here we showed that BPV L1 VLPs can also be disrupted and reassembled in vitro. Since VLPs are non-infectious, we showed that reassembled VLPs regained the ability to recognize their cellular receptor by haemagglutination of mouse red blood cells. This HA efficiency was 4-fold less of the untreated VLPs, pointing towards a decrease in the amount of reassembled particles recovered.

Based on our results we hypothesize that calcium plays a role in the stabilization of BPV virions. In order to transport the viral DNA into the target cell nucleus during infection, the capsid must disintegrate. For BPV, this process is not yet understood. One possible mechanism could be the removal of calcium ions from the capsid structure which leads to capsid disintegration, as shown for many plant viruses (Durham & Haidar, 1977). Addition of calcium ions to the viral capsid must occur during virus assembly. Assembly of papillomavirus particles occurs in the nucleus of infected host cells (Orth et al., 1977). Calcium ions are constantly and rapidly shuttled in and out of the nucleus (Allbritton et al., 1994). These fluctuations coupled with other factors present in the cells may control the
incorporation and removal of calcium ions in BPV virions during assembly in the nucleus and uncoating in the cytoplasm respectively.

We thank Drs R. Roden and J. Schiller (NIH) for providing us with anti-BPV-1 L2 antibody. The bovine warts were kindly provided by Drs R. Lowe (Merck, PA) and P. Lambert (University of Wisconsin–Madison, WI). We thank J. Brainer and E. Sundin of the Pathology department for technical assistance with the electron microscope. We acknowledge the help of Dr C. Walz with this manuscript. This project was supported in part by the Schweppes Foundation, NH&MRC and Queensland Cancer Fund.

References


Received 28 October 1997; Accepted 19 January 1998