Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic

R. Kirnbauer*, F. Booy†, N. Cheng†, D. R. Lowy*, and J. T. Schiller*

*Laboratory of Cellular Oncology, National Cancer Institute, Bethesda, MD 20892; and †Laboratory of Structural Biology Research, National Institute of Arthritis, Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892

Communicated by Hidesaburo Hanafusa, September 22, 1992 (received for review August 25, 1992)

ABSTRACT Infection by certain human papillomavirus types is regarded as the major risk factor in the development of cervical cancer, one of the most common cancers of women worldwide. Analysis of the immunogenic and structural features of papillomavirus virions has been hampered by the inability to efficiently propagate the viruses in cultured cells. For instance, it has not been established whether the major capsid protein L1 alone is sufficient for virus particle assembly. In addition, it is not known whether L1, L2 (the minor capsid protein), or both present the immunodominant epitopes required for induction of high-titer neutralizing antibodies. We have expressed the L1 major capsid proteins of bovine papillomavirus type 1 and human papillomavirus type 16 in insect cells via a baculovirus vector and analyzed their conformation and immunogenicity. The L1 proteins were expressed at high levels and assembled into structures that closely resembled papillomavirus virions. The self-assembled bovine papillomavirus L1, in contrast to L1 extracted from recombinant bacteria or denatured virions, also mimicked intact bovine papillomavirus virions in being able to induce high-titer neutralizing rabbit antisera. These results indicate that L1 protein has the intrinsic capacity to assemble into empty capsid-like structures whose immunogenicity is similar to infectious virions. This type of L1 preparation might be considered as a candidate for a serological test to measure antibodies to conformational viral epitopes and for a vaccine to prevent papillomavirus infection.

Papillomaviruses infect the epithelia of humans and a wide variety of animals, where they generally induce benign proliferation at the site of infection. However, in some cases the lesions induced by certain papillomaviruses undergo malignant progression. There is a strong association between malignant progression of human genital lesions and certain human papillomavirus (HPV) types, such as HPV16. Infection by one of these types is considered the most significant risk factor in the development of cervical cancer, one of the most common cancers of women worldwide (for review, see refs. 1 and 2). The majority of cervical carcinomas contain and express HPV early genes, and these genes have been shown to have potent transforming and immortalizing activity in cultured cells (for review, see ref. 3).

The papillomavirus oncoproteins and other nonstructural proteins are expressed in cultured cells and have been extensively studied in vitro. However, the structural viral proteins L1 and L2 are not normally expressed in culture, a fact that has slowed progress in characterizing these proteins and inhibiting the propagation of papillomaviruses in cultured cells. In vivo, expression of L1 and L2 and the assembly of infectious virions only occur in terminally differentiated layers of epithelial tissues (for review, see ref. 4). All papillomavirus virions are nonenveloped 50- to 60-nm icosahedral structures (5) that are comprised of conserved L1 major capsid and less-well-conserved L2 minor capsid proteins (6). The recent high-resolution cryo-electron microscopic analysis of bovine papillomavirus type 1 (BPV1) and HPV1 virions has determined that the two viruses have a very similar structure, with 72 pentameric capsomeres, each presumably composed of five L1 molecules, forming a virion shell with \( T = 7 \) symmetry (7). Superficially, this arrangement resembles the structure of the more extensively studied 45-nm virions of the polyomaviruses, which also have the same number of capsomeres and symmetry (8, 9). However, the system of intercapsomer contacts is different for the two groups of viruses and the major and minor polyomavirus capsid proteins are not genetically related to L1 or L2. The location of the minor capsid L2 protein in the virion has not been determined, and it is not known whether L2, or other viral proteins, are required for capsid assembly.

Relatively little is known about the immunity to papillomavirus infections, in large part due to the inability to generate virus stocks in vitro. Studies of immunity to papillomavirus infection have principally been conducted using the BPV model, since relatively large numbers of infectious virus particles can be isolated from BPV-induced warts, and, in contrast to the HPV's, a quantitative focal transformation assay for BPV infectivity has been developed (10). Rabbit antisera raised against infectious BPV1 virions have been shown to contain high-titer antibodies that inhibit focal transformation of C127 cells (10) and also inhibit transformation of fetal bovine skin grafts, whereas antisera raised against denatured virions do not (11). In addition, monoclonal antibodies (mAbs) that neutralized HPV11 infection in a mouse xenograft assay recognized native, but not denatured, HPV11 virions (12). Therefore, it appears that neutralizing antibodies recognize conformationally dependent epitopes. It has been reported that neutralizing antibodies can be generated against both bacterially derived BPV L1 and L2 but these antibodies were of very low titer (13, 14). Similarly, in vitro-synthesized ctenontail rabbit papillomavirus L1 and L2 only induced low-titer neutralizing sera (15, 16). It is not therefore known whether the high-titer antibodies raised against intact virions principally recognize epitopes from L1, L2, or a combination of the two proteins.

With the long-term goal to gain insight into the immune response to papillomavirus infection and to develop a papillomavirus vaccine, we have expressed the L1 major capsid protein of HPV16 and BPV1 in insect cells. In contrast to previous reports (17), we demonstrate that L1 alone is sufficient for assembly of virus-like particles that are morphologically similar to native virions. These in vitro-synthesized particles are able to induce high-titer neutralizing antisera that are capable of preventing papillomavirus infection in vivo.

Abbreviations: BPV, bovine papillomavirus; HPV, human papillomavirus; AcMNVP, Autographa californica multiple nuclear polyhedrosis virus; nt, nucleotide(s); mAb, monoclonal antibody; wt, wild type.
MATERIALS AND METHODS

Cell Culture and Virus Stocks. Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) and recombinant viruses were propagated as described (18). SF9 cells (ATCC, CRL 1711) were maintained in Grace's insect medium supplemented with lactalbumin (3.33 mg/ml), yeastolate (3.33 mg/ml), and 10% (vol/vol) fetal calf serum (GIBCO) in a 27°C incubator.

Construction of Transfer Vectors Containing the HPV16-L1 and BPV1-L1 Genes and Selection of Recombinant Baculoviruses. The L1 open reading frames were amplified by the PCR using the cloned prototype of BPV1 (19) or HPV16 (20) DNA as templates. Unique restriction sites were incorporated into the oligonucleotide primers. BPV1-L1 primer sequence, 5'-CCGGCTGAATCTAATATGCCGTGGCCAAAGCCGAGTGTAT-3' (sense) and 5'-CCGGTGGTACCTGCAATGTTGTCCTACCTCTGTATTTTACATTACAGC-3' (antisense); HPV16-L1 primer sequence, 5'-CCGGCTAGATCTAATATCTTGTCCCTTGCGCTGGCTAGTAGGGGCCC (sense) and 5'-CCGGTGGATCTAGCTATCCAATACATACATAATCCCTACGC-3' (antisense). L1 coding sequences begin at the first methionine codon (boldface). Viruses for BPV1-L1 and the second methionine for HPV16-L1 were cloned as a 5' EcoRI to 3' Kpn I fragment and HPV16-L1 was cloned as a 5' Bgl II to 3' Bgl II fragment into the multiple cloning site downstream of the polyhedrin promoter of the AcMNPV-based baculovirus transfer vector pEVmod (21) and amplified by PCR.

Expression of L1 Proteins in Insect Cells. SF9 cells were either mock-infected or infected at a multiplicity of infection of 10 with wt AcMNPV or AcBPV-L1 or AcHPV16-L1 recombinant virus. After 72 h, cells were lysed by boiling in Laemmli buffer, and the lysates were subjected to SDS/PAGE in 10% gels. Proteins were either stained with 0.25% Coomassie blue or analyzed on an immunoblot probed with BPV-L1 mAb AU-1 (23) or HPV-L1 mAb CAMVIR-1 (24) and 125I-labeled Fab anti-mouse IgG (Amersham).

Purification of Particles and Transmission Electron Microscopy Analysis. SF9 cells (500 ml; 2 x 10^6 cells per ml) were infected with AcBPV-L1 or AcHPV16-L1 recombinant baculoviruses. After 72 h, the harvested cells were sonicated in phosphate-buffered saline (PBS) for 60 sec. After low-speed clarification, the lysates were subjected to centrifugation at 110,000 x g for 2.5 h through a 40% (wt/vol) sucrose/PBS cushion (SW-28 rotor). The resuspended pellets were centrifuged to equilibrium at 141,000 x g for 20 h at room temperature in a 10-40% (wt/wt) CsCl/PBS gradient. Fractions were harvested from the bottom and analyzed by SDS/PAGE. Immune reactive fractions were dialyzed against PBS, concentrated by Centri concentrate-30 (Millipore) ultrafiltration, and for HPV16-L1 pellet by centrifugation for 10 min at 30 psi (1 psi = 6.9 kPa) in an A-100 rotor in an Airfuge (Beckman). BPV1 virosomes were purified from a bovine wart (generously provided by A. B. Jenson, Georgetown University, Washington, DC) as described (25). Purified particles were adsorbed to carbon-coated grids, stained with 1% uranyl acetate, and examined with a Philips electron microscope model EM 400T at x 36,000 magnification.

Production of Antisera. Rabbits were immunized by subcutaneous injection of whole-cell SF9 lysates (3 x 10^7 cells) prepared by one freeze/thaw cycle and 20 strokes in a Dounce homogenizer (rabbits 1, 2, and 8) or 200 µg of L1 protein partially purified by 35% (saturation at 4°C) ammonium sulfate precipitation (rabbits 3, 4, 6, and 7), in complete Freund's adjuvant, and then given two booster injections at 2-week intervals, with the same preparations in incomplete Freund's adjuvant.

BPV1 Neutralization Assay. Serial dilutions of sera obtained 3 weeks after the second booster injection were incubated with 500 focus-forming units of BPV1 virus for 30 min, the virus was absorbed to C127 cells for 1 h, and the cells were cultured for 3 weeks (10). The foci were stained with 0.5% methylene blue/0.25% carbol fuchs in methanol.

RESULTS

Expression and Characterization of L1 Major Capsid Protein. To begin a study of the structural and immunogenic features of papillomavirus virions, we sought to generate renewable reagents in a eukaryotic system by amplifying the complete L1 gene of BPV1 by PCR and cloning it into an AcMNPV baculovirus-based transfer vector. The L1 open reading frame was placed under the control of the baculovirus polyhedrin promoter. After cotransfection of the L1 clone with the wt baculovirus DNA into SF9 insect cells and plaque purification of recombinant clones, high-titer recombinant virus was generated. Extracts from cells infected with wt AcMNPV or BPV1 recombinant viruses (AcBPV-L1) were analyzed by PAGE. After Coomassie blue staining, a unique protein of the predicted size, 55 kDa, was detected in the extracts from the cultures infected with the AcBPV-L1 virus (Fig. 1A). The identity of this protein as BPV L1 was verified by immunoprecipitation (data not shown) and immunoblot analysis (Fig. 1B) using the BPV-L1-specific mAb AU-1 (23).

Although we were concerned that L2 and/or specific factors provided by differentiated epithelial cells might be required for assembly of papillomavirus virions, the possibility existed that papillomavirus L1 has the ability to self-assemble into virus-like particles when overexpressed in heterologous cells. We were encouraged by previous reports that the major capsid proteins of several other groups of viruses, such as hepatitis B (26), polyoma (27), and parvovirus (28), can self-assemble. We therefore examined elec-

Fig. 1. Expression of L1 proteins in insect cells. SF9 cells were either mock-infected (lanes mock) or infected at a multiplicity of infection of 10 with wt AcMNPV (lanes wt) or AcBPV-L1 (lanes B-L1) or AcHPV16-L1 (lanes 16-L1) recombinant virus. After 72 h, cells were lysed by boiling in Laemmli buffer and the lysates were subjected to SDS/PAGE in 10% gels. Proteins were either stained with 0.25% Coomassie blue (A) or analyzed on an immunoblot probed with BPV-L1 mAb AU-1 (B) or HPV-L1 mAb CAMVIR-1 (C) and 125I-labeled Fab anti-mouse IgG (Amersham). P designates polyhedrin protein. Molecular masses in kDa are shown.
 localized in the nucleus of cells (data not shown). These circular structures of particles results suggested that self-assembly of particles had occurred, since in vivo papillomavirus virion assembly takes place in the nucleus and the diameter of the virions has been reported as 55 nm.

**Purification of Virus-Like Particles and Transmission Electron Microscopy.** To obtain further evidence that the L1 protein had self-assembled, we purified virus-like particles from the infected insect cells by using gradient centrifugation. High molecular mass structures were separated from lysates of L1 recombinant or wt infected cells by centrifugation through a 40% sucrose cushion and the pelleted material was subjected to CsCl density gradient centrifugation. Fractions were collected and tested for reactivity to the BPV-L1-specific mAb by immunoblot analysis.

L1-positive fractions from the gradient were adsorbed onto carbon film grids, stained with 1% uranyl acetate, and examined by transmission electron microscopy. The positive fractions contained numerous circular structures that exhibited a regular array of capsomers (Fig. 2A). Most were ≈50 nm in diameter, although smaller circles and partially assembled structures were also seen. The larger particles were very similar in size and subunit structure to infectious BPV virions that had been stained and photographed concurrently (Fig. 2B). Consistent with previous reports of the density of empty BPV virions (29), the density of the CsCl fraction containing the virus-like particles was ≈1.30 g/ml. These particles were not observed in preparations from mock-infected or wt-AcMNPV-infected cells (data not shown). From these results, we conclude that BPV1-L1 has the intrinsic capacity to assemble into virus-like particles in the absence of L2 or other papillomavirus proteins.

**Generation of High-Titer Neutralizing Antisera.** Previous attempts to generate high-titer neutralizing antisera against BPV using L1 extracted from bacteria have not been successful. Since intact infectious virions, but not denatured virions, are able to induce high-titer neutralizing antibodies, it is likely that the bacterially derived L1 was weakly immunogenic because it had not assumed a native conformation and/or had not assembled into virion-like structures. Also, multiple electrophoretic variants of L1 have been detected in virions (29). Some of these modified species, which are probably absent in the bacterially derived L1, may facilitate the generation of neutralizing antibodies. We were therefore interested in determining whether the assembled BPV L1 from insect cells could induce neutralizing antisera in rabbits. Two types of preparations were tested: whole-cell extracts of L1 recombinant- or wt-virus-infected Sf9 cells and partially purified particles, which were isolated by differential centrifugation and ammonium sulfate precipitation. The rabbits received a primary inoculation followed by two booster injections at 2-week intervals.

The sera were tested for the ability to inhibit BPV infection of mouse C127 cells, as measured by a reduction in the

![Figure 2](image_url)
number of foci induced by a standard amount of BPV virus. A representative assay is shown in Fig. 3. The immune sera generated by inoculation with baculovirus-derived L1 were able to reduce the infectivity of the BPV by 50% at a dilution of at least 1:11,000 (a titer of 11,000), whereas the preimmune sera from the same rabbits did not inhibit focal transformation at a dilution of 1:20, the lowest dilution tested (Table 1). Both the crude preparations and partially purified particles were effective in inducing high-titer neutralizing antisera, with 290,000 being the highest titer measured. This was the same as the neutralizing titer of the positive control antiserum raised against infectious BPV virions. In comparison, the highest titer generated in a previous study using bacterially derived L1 was 36 (13). The control serum from a rabbit inoculated with the extract from wt-baculovirus-infected cells was unable to inhibit infectivity at a dilution of 1:20, indicating that the neutralizing activity was L1-specific. Disruption of the partially purified L1 particles by boiling in 1% SDS abolished the ability of the preparation to induce neutralizing antibodies (Table 1).

From the above results, we conclude that the L1 particles assembled in insect cells mimic infectious virus in the presence of antibodies specific for L1.

![Fig. 3. BPV1 neutralization assay. Serial dilutions of sera obtained 3 weeks after the second booster injections were incubated with BPV1 virus, the virus was absorbed to C127 cells, and the foci were stained after culturing the cells for 3 weeks (10). The antisera and dilutions used are indicated below the plates. Anti-AcBPV-L1 was obtained from rabbit 1 and anti-wt AcMNPV was from rabbit 8 (Table 1). n.r.s. is the normal rabbit serum negative control, anti-BPV-1 virion was raised against native BPV virions (23), and Dako is the commercially available (DAKO, Carpinteria, CA) rabbit antiserum raised against denatured BPV virions.](image)

Table 1. BPV1 neutralizing antibody titer

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Rabbit</th>
<th>Serum neutralization titer against BPV1</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcBPV-L1</td>
<td>1</td>
<td>11,000</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>97,000</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>290,000</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>97,000</td>
</tr>
<tr>
<td>BPV1 virions*</td>
<td>5</td>
<td>290,000</td>
</tr>
<tr>
<td>AcBPV-L1/SDS</td>
<td>6</td>
<td>&lt;2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>&lt;2</td>
</tr>
<tr>
<td>wt AcMNPV</td>
<td>8</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

The titer is the reciprocal of the dilution that caused 50% focus reduction. Assays were carried out as in Fig. 3. Rabbits 1, 2, and 8 were inoculated with crude whole-cell SF9 lysates, and rabbits 3, 4, and 7 were inoculated with partially purified L1 protein. Rabbits 6 and 7 were immunized with L1 protein preparations that had been denatured by boiling in 1% SDS. At least two sera, taken 3–6 weeks after the second booster injection, were tested for each rabbit and found to have the same titer. The titer of the preimmune sera from each of the rabbits was <20, the lowest dilution tested.

*Provided by A. B. Jenson and purified as described (23).

sentation of conformationally dependent immunodominant epitopes. These results also establish that L2 is not required for the generation of high-titer neutralizing antibodies.

To determine whether the ability to self-assemble in insect cells is a general feature of papillomavirus L1, we also expressed the L1 of HPV16, the HPV type most often detected in human genital cancers, via an analogous recombinant baculovirus. A 58-kDa protein, the expected size, was expressed at high levels in the insect cells infected with the HPV16 L1 recombinant virus (Fig. 1A) and it reacted strongly with an HPV16 L1 mAb (which also cross-reacted weakly with BPVL1; Fig. 1C). After CsCl gradient purification, immunoreactive fractions were examined by electron microscopy and found to contain 50-nm papillomavirus-like particles (Fig. 2C). In comparison to the BPV L1 preparations, fewer completely assembled particles were seen. This may be due to the lower levels of expression and/or greater extent of HPV16 L1 degradation (Fig. 1). We conclude that the L1 of HPV16, and presumably the L1 proteins of other HPV types, also has the ability to self-assemble into virion-like structures.

**DISCUSSION**

In this study we have shown that L1, the major capsid protein of HPVs and BPVs, has the intrinsic capacity to self-assemble into virus-like particles in the absence of L2 or other papillomavirus proteins. These particles are similar to native virions in morphology. Our results differ from those of a recent study (17) that found that simultaneous expression of HPV16 L1 and L2 resulted in formation of smaller 35- to 40-nm particles but that particles were not observed when HPV16 L1 alone was expressed. One possible interpretation might be that L2 facilitates the assembly of particles, and the vaccinia-based expression system used in that study did not express L1 at high enough levels to allow for efficient self-assembly. Our results indicate that specific factors limited to differentiating epithelia or mammalian cells are not required for papillomavirus capsid assembly. Since the baculovirus-based assembly system involves acute infection of cells and permits the genetic manipulation of the L1 gene, it should now be possible to study the process of papillomavirus capsid formation and to determine the L1 domains involved in self-assembly.

Two recent studies have demonstrated that certain HPV-containing epithelial cell lines express the late proteins and produce virion-like particles when they are induced to dif-
ferentiate in a culture (30, 31). These systems will be useful in elucidating the specific cellular signals that control viral late gene expression. However, it is not practical to produce preparative amounts of viral particles with raft cultures and, since the viral genomes are not currently amenable to genetic manipulation, the potential usefulness of these systems in the analyses of virion assembly is unclear. We have demonstrated that L1 papillomavirus-like particles engineered and expressed in insect cells induce neutralizing antibody titers at least 1000-fold higher than previously achieved. These results also establish that L2 is not required for the generation of high-titer neutralizing antibodies. This observation is in contrast to recent studies of immunity to human B19 parvovirus infection. Parvovirus VP2 alone was able to self-assemble when expressed in insect cells but only particles containing both VP1 and VP2 were able to induce neutralizing antibodies (28).

Previous serologic studies of human immune response to papillomavirus virion proteins have principally utilized bacterially expressed L1 and L2 capsid proteins (32), and the results have not correlated well with other measures of HPV infection. However, the BPV studies discussed above indicated that papillomavirus virion proteins extracted from bacteria do not present the conformationally dependent epitopes that appear to be type specific and recognized by most neutralizing antibodies. Therefore, self-assembled L1 papillomavirus-like particles could be used to develop a serological test to determine the extent of anti-HPV virion immunity in human populations. Such a test, which could measure neutralizing antibodies, is likely to be more accurate and biologically relevant than assays that primarily recognize linear epitopes.

Subunit vaccines, based on self-assembled virion proteins synthesized in heterologous cells, have proven to be safe and effective in preventing infections by several pathogenic viruses, including human hepatitis B (33). The limited studies using intact BPV virions as a vaccine suggest that papillomavirus virion proteins can generate a protective immune response. Noncutaneous inoculation of infectious or formalin-inactivated BPV has been reported to be effective as a vaccine to prevent experimental BPV infection in calves (34, 35). BPV-transformed cells were ineffective, suggesting that viral capsid proteins, rather than early gene products, elicited the protective response. Bacterially derived L1 was able to generate partial protection against experimental challenge with BPV, despite the low titer of neutralizing antibodies induced by these preparations (36–38). Comparable results have recently been obtained for cottontail rabbit papillomavirus using L1 (and L2) extracted from bacteria or vaccinia-infected cells (16). The above results are encouraging in that they indicate that L1 can induce protection against papillomavirus infection under controlled experimental conditions in which a standard inoculum of virus is administered soon after vaccination. Our results demonstrate that L1 can self-assemble into virion-like particles that elicit neutralizing antisera titers that are at least three orders of magnitude higher than were previously obtained with in vitro-produced antigens. This suggests that this type of preparation may have the potential for a subunit vaccine to induce effective long-term protection against naturally transmitted papillomavirus infection.

We thank A. B. Jenson for generously providing a BPV1 papilloma, mAb AU-1, and the BPV1-neutralizing antisera; C. McLean for mAb CAMVIR-1, and L. Miller for pEVMod. R. K. is supported by a Schrödinger scholarship from the Austrian Forschung zur Förderung der wissenschaftlichen Forschung (J0681-MED).