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Human Papillomavirus Type 16 Minor Capsid Protein L2 N-Terminal Region Containing a Common Neutralization Epitope Binds to the Cell Surface and Enters the Cytoplasm

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The first step of papillomavirus infection is believed to be binding of major capsid protein L1 to the cell surface without involvement of minor capsid protein L2, but the viral infectivity can be neutralized either by anti-L1 or anti-L2 antibody. To understand the role of L2 in human papillomavirus (HPV) infection, we examined a segment of HPV type 16 (HPV16) L2, which contains a neutralization epitope common to HPV6, for its involvement in adsorption and penetration of the capsids. Preincubation of monkey COS-1 cells with a synthetic peptide having amino acids (aa) 108 to 120 of HPV16 L2 reduced the susceptibility of COS-1 cells to infection with HPV16 pseudovirions. Confocal microscopy showed that the green fluorescence protein (GFP) fused with the L2 peptide was found to bind to the surface of a HeLa cell, an HPV18-positive human cancer cell line, at 4°C and to enter the cytoplasm after subsequent incubation at 37°C. Flow cytometry showed that fused GFP did not bind to HeLa cells that had been treated with trypsin. Besides COS-1 and HeLa cells, some human and rodent cell lines were detected by flow cytometry to be susceptible to binding with fused GFP, showing a tendency of epithelial cells toward higher susceptibility. Substitutions at aa 108 to 111 inhibited fused GFP from binding to HeLa cells and reduced the infectivity in COS-1 cells of the in vitro-constructed pseudovirions. The results suggest that L2 plays an important role in enhancing HPV infection through interaction between the N-terminal region and a cellular surface protein, facilitating penetration of the virions and determining part of the tropism of HPVs.

Papillomaviruses, whose nonenveloped, icosahedral, 55-nm-diameter virions are made of 72 pentameric capsomeres composed of the structural proteins L1 and L2 at an estimated molar ratio of 30 to 1, have been found in various animal species, including humans (26). Human papillomaviruses (HPVs) have to date been classified into more than 80 genotypes, which constitute two groups: cutaneous and mucosal HPVs (26). HPVs infecting the cutaneous epithelium such as types 1, 2, 4, and 8 mainly cause skin warts (26). HPVs infecting the mucosal epithelium such as types 6 and 11 cause benign condyloma, but types 16, 18, and 33 cause cervical cancer (14, 26). Among the nine major types of HPV associated with cervial cancer, HPV16 is the most prevalent type, constituting approximately 50% of cases (14, 27).

Because it is almost impossible to efficiently grow HPVs in cell cultures, except HPV18 in a raft culture (16), surrogate systems have been developed for production of capsids (particles without viral DNA) and for assay of viral infectivity. The capsids produced in surrogate systems resemble morphologically and immunlogically the natural virions (7, 11, 12, 22). When L1 alone is expressed in eukaryotic cells by recombinant baculovirus or vaccinia virus (7, 11, 12, 22), L1 can self-assemble to form icosahedral particles (L1 capsids). When L2, which is not required for assembly, is coexpressed with L1, both L1 and L2 are incorporated into the particles (L1-L2 capsids) (7, 12). Furthermore, infectious HPV pseudovirions are produced in cultured cells (19, 24). Also, pseudovirions are constructed in vitro from disassembled capsids and a plasmid capable of expressing a reporter gene, and their infectivity can be assayed in COS-1 cells (9). The capsids and pseudovirions and natural bovine papillomavirus type 1 (BPV1) virions, isolated from cutaneous legions, have been used for studies of adsorption of papillomaviruses and of neutralization of their infectivity.

Viral infection in vitro is supposed to start from attachment of virions to the cell surface (20, 25). The adsorption appears to occur from binding of L1 to cell receptors without involvement of L2, because virions of BPV1 (20) and L1-L2 and L1 capsids of BPV1, HPV11, HPV16, and HPV33 (17, 20, 25) are seemingly capable of binding to cells with similar efficiencies and because a mouse anti-BPV1 L2 monoclonal antibody, which inhibits the infectivity of BPV1 (focus formation in mouse C127 cells), allows binding of BPV1 virions to C127 cells (18). Thus, L2 may not be a major factor required for adsorption, but it appears to affect infectivity, presumably at a postadsorption step, based on the findings that infectivity is higher with the L1-L2 pseudovirons than with the L1 pseudovirons (9, 24). Although it is not yet clear how L2 affects infectivity, studies of neutralization with anti-L2 antibodies (1, 3, 4, 6, 10, 13, 21) suggest that the initial interaction between L2 and the cell surface is important.

We have shown that anti-L2 antibodies that inhibit HPV16

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pseudovirion infection recognize a linear epitope localized within the L2 N-terminal region from amino acids (aa) 108 to 120 [L2(108–120)] (8, 10). The amino acid sequence of this region is highly conserved in other mucosal HPVs (Sequence Database of Los Alamos National Laboratory [10]), and the neutralization epitope is common to HPV16 and HPV6 (10). It is likely, therefore, that this L2 region has an important function in HPV infection.

To understand the role and behavior of L2 in HPV infection, we examined the HPV16 L2 region from aa 108 to 126 [L2(108–126)] for its interaction with cells with regard to infectivity in this study. A competition assay showed that the L2(108–120) peptide interfered with the infectivity of HPV16 pseudovirions in COS-1 cells, suggesting the presence of a receptor for L2 on the cell surface. Experiments using green fluorescence protein (GFP) (2) fused with L2(108–126) indicated the attachment of the peptide on the cell surface and its entry to the cytoplasm. The pseudovirions containing mutated L2 that lacked the ability to bind showed reduced infectivity, suggesting that the binding of L2 to the surface protein is required for efficient HPV infection.

MATERIALS AND METHODS

Cells. Human cell lines derived from cervical cancer (HeLa, SiHa, and CaSki) and from liver cancer (HepG2 and Alexander), a human cell line transformed with the adenovirus E1 gene (293), a monkey epithelial cell line (COS-1), and rodent cell lines (C127, NIH 3T3, and 3Y1) were cultured in Dulbecco's modified Eagle's medium (GIBCO BRL, New York, N.Y.) with 10% fetal calf serum (FCS) at 37°C and with 5% CO_2. The mouse myeloma cell line PAI was cultured in RPMI 1640 (GIBCO BRL) with 10% FCS. For suspension culture, HeLa cells were grown in S-MEM minimum essential medium (GIBCO BRL) in spinner flasks. A insect cell line (Sf9) was grown at 27°C in optimized serum-free medium (Sf-900 II SFM; GIBCO BRL) supplemented with 3% FCS.

Construction of pseudovirions and infectivity assay. Infectious pseudovirions were constructed in vitro through reassembly of disassembled L1 or L1-L2 capsids in the presence of a plasmid capable of expressing β-galactosidase as described previously (9). The pseudovirions were allowed to infect COS-1 cells, and infectivity was measured by counting blue cells as described previously (9). For large-scale preparation of capsids, Sf9 cells infected with the recombinant baculovirus were cultured by using a cell culture controller, CELLMASTER model 1700 (Wakenyaku, Kyoto, Japan). DNase I-resistant plasmid DNA in pseudovirions was measured by quantitative PCR with an ABI PRISM7700 sequence detector (PE Applied Biosystems, Foster City, Calif.). Since infectivity of pseudovirions declined during storage at 4°C, pseudovirions were freshly prepared before use in this study.

Pseudovirions containing L2 with amino acid substitutions were newly constructed. The DNA fragment encoding the HPV16 L2 region of aa 99 to 124, which contained an amino acid substitution of GGDD for LVEE, was generated by PCR using the primers 5′-GGGCCCTTCTGATCCTTCTATAGTTTCTGG TGGTGATGATACTAGT-3′ and 5′-GGGCCCAGGTACAGATGTTGGTGC ACC-3′ and a plasmid for GFP-L2(108–126), which contained an amino acid substitution of GGDD for LVEE as a template. The resultant DNA was inserted into the complete L2 fragment of the pUC/HPV16L2 plasmid at the *Apa*I site. Then, an *Eco*RI fragment of 1.5 kb was isolated and inserted into pEGFP-C1 (Clontech Laboratories, Inc., Palo Alto, Calif.) at its *Eco*RI site. After digestion with *Apa*LI, plasmid DNA was diluted and self-ligated to remove the shortest *Apa*LI fragment. The total L2 region with mutations was inserted between the *Sma*I and *Xho*I sites of the previously constructed pBacDual vector for HPV16 L1 and L2 (8). A recombinant baculovirus for L1-L2 capsids containing L2 with the substitution were generated, and pseudovirions were constructed in vitro through reassembly of disassembled L1-L2 capsids in the presence of a plasmid capable of expressing β-galactosidase as described previously (9).

GFPs fused with HPV16 L2 segment aa 108 to 126. The GFP gene (2) was amplified by PCR from phGFP-S65T(Clontech Laboratories, Inc.) using the sense primer 5′-GGATCCATGGTGAGCAAGGGCGA-3′ and the antisense primer 5′-AAGCTTTTACTTGTACAGCTCGTCCATGCC-3′. Three sense primers containing HPV16 nucleotides (Sequence Database of Los Alamos National Laboratory) 358 to 378, 343 to 375, and 322 to 359 in their 5′ regions (5′-CCAACATCTGTACCTTCCATTATGGTGAGCAAGGGCGA-3′, 5′-AT TGATGCTGGTGCACCAACATCTGTACCTTCC-3′, and 5′-GGATCCTTA GTGGAAGAAACTAGTTTTATTGATGCTGGTGCACC-3′) and the antisense primer were used for three successive PCRs to generate a DNA fragment encoding GFP-L2(108–126). DNA fragments encoding mutated GFP-L2(108–126) were constructed similarly using a DNA fragment of GFP-LP(108–126) for the template and the antisense primer used to generate GFP-LP(108–126). The sense primers 5′-ATTGATGCTCTGCTGCCAACATCTGTACCTTCC-3′ and 5′-GGATCCTTAGTGGAAGAAACTAGTTTTATTGATGCTCTGCTGC C-3′ were used to substitute LL for GA; the primers 5′-ATTGATGCTCCACC ACCAACATCTGTACCTTCC-3′ and 5′-GGATCCTTAGTGGAAGAAACT AGTTTTATTGATGCTCCACCACC-3′ were used for substitutions of PP for GA; and the primer 5′-GGATTCGGTGGTGATGATACTAGTTTTATTGAT GCTGGTGCACCAACATC-3′ was used for substitutions of GGDD for LVEE. The amplified DNA was subcloned into pGEMT (Promega Corp., Madison, Wis.), and the structure of the plasmid was verified by DNA sequencing. The DNA fragments encoding GFP or GFP-L2(108–126) were inserted into the pFastBacHT donor plasmid (GIBCO BRL) between the *Bam*HI and *Hin*dIII sites, and the resultant plasmid was introduced into DH10Bac competent bacterial cells to obtain Bacmid DNA. The recombinant baculovirus propagated in Sf9 cells that were transfected with Bacmid DNA generated in DH10Bac. Histidine-tagged GFPs expressed in Sf9 cells were purified from cell lysate using an Ni column (GIBCO BRL) according to the manufacturer's standard protocol. The purity and concentration of GFPs were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All GFPs used in this study gave strong fluorescence under UV radiation with similar efficiencies.

Confocal microscopy. To HeLa and the other cells (2×10^5) grown on a glass slide (Lab-Tek chamber slide; Nalge Nunc International Corp., Naperville, Ill.) was added GFP or GFP-L2(108–126) at 4 μg/ml in plain Dulbecco's modified Eagle's medium after removal of the culture medium. The cells were allowed to react with the GFPs at 4°C for 1 h and washed with plain medium twice for removal of unbound GFPs. In some cases the cells were incubated with GFP or GFP-L2(108–126) at 4°C for 1 h, washed twice, and further incubated in culture medium at 37°C for 2 h. Then, the cells that had been allowed to react with GFPs were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min at 37°C, permeated in PBS containing 0.1% Triton X-100 for 10 min, and washed three times with TBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl). To stain nuclei, the slide cultures were soaked with 20 mg of propidium iodide per ml for 10 min and washed with TBS three times. Fluorescence was examined with a model LSM510 laser scanning system (Carl Zeiss Co. Ltd., Oberkochen, Germany).

Assay for binding of GFPs to the cell surface by fluorescence-activated cell sorter analysis. Cells grown in a culture plate were dispersed with PBS containing EDTA (2.5 mM) and washed with S-MEM. HeLa cells in suspension culture were collected by centrifugation. Cells (4×10^5) were resuspended in 2 ml of plain S-MEM containing GFP, GFP-L2(108–126), or other fused GFPs (4 μg/ml); incubated at 4°C for 1 h; and washed twice. The fluorescence of cells was analyzed with a FACSCalibur (Becton Dickinson Immunocytometry Systems, Inc., Franklin Lakes, N.J.).

RESULTS

Lowered susceptibility of COS-1 cells preincubated with HPV16 L2(108–120) to infection with pseudovirions. In an attempt to visualize the interaction of the L2 peptide that contains a common neutralization epitope with cells, we prepared GFP-L2(108–126) (Fig. 1). Preliminary experiments by confocal microscopy showed that the GFP fusion peptide bound to the surfaces of COS-1 cells, a monkey epithelial cell line positive for simian virus 40 T antigen that has been used for an HPV pseudovirion infection assay (9), and HeLa cells, a cell line derived from HPV18-positive human cervical cancer. To correlate peptide binding with pseudovirion infection, we tested whether the peptide affects infection by competing with L2 in virions for binding to the surfaces of COS-1 cells.

COS-1 cells (4×10^5) were dispersed with EDTA-PBS, washed with PBS once, and incubated at 4°C with occasional agitation in 80 μl of PBS containing none or one of the following competitors: P-108/120 [a synthetic peptide with the

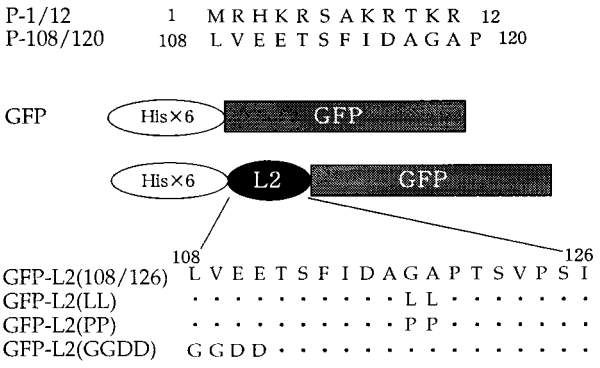

FIG 1. Schematic representation of synthetic peptides and GFP-L2 fusion proteins used in this study. HPV16 L2 amino acids are deduced from the HPV16R nucleotide sequence (Sequence Database of Los Alamos National Laboratory). The entire L2 peptide consists of 473 aa. GFP-L2 fusion proteins were expressed in insect Sf9 cells by recombinant baculoviruses and purified by affinity column chromatography. GFP is composed of 238 aa (2). His × 6, six histidines.

sequence of HPV16 L2(108–120)], P-1/12 (a peptide with the sequence of HPV16 L2 from aa 1 to 12), GFP, or GFP-L2 (108–126). One hour later, the cells were infected with L1-L2 or L1 pseudovirions, incubated another hour, and then seeded in the growth medium. Inoculum used for each infection contained 100 ng of L1. Cells expressing β-galactosidase were counted after an incubation of 48 h.

Peptide P-108/120 and the fusion protein GFP-L2(108–126) were found to interfere with the infectivity of L1-L2 pseudovirions but not that of L1 pseudovirions (Fig. 2). By preincubation of COS-1 cells with P-108/120 or GFP-L2, the number of blue cells produced by infection with L1-L2 pseudovirions was dropped to less than half of that obtained by preincubation without the peptide. Since incubation with P-1/12 or plain GFP did not reduce the number of blue cells, the reduction required the amino acid sequence of the L2 peptide. The numbers of blue cells produced by infection with L1 pseudovirions, which were less infectious than L1-L2 pseudovirions, were not influenced by preincubation with the L2 peptide. The data indicate that the peptide containing the common neutralization epitope competes with the L2 region displayed on the surfaces of HPV virions for binding to a cellular surface protein, suggesting that the binding to the cellular target is important for L2 to enhance infectivity. For the cells whose surface targets for L2 were saturated with P-108/120 or GFP-L2(108–126), L1-L2 pseudovirions may have lost the advantage of having L2 and behaved like L1 pseudovirions in the infectivity assay.

Binding of the L2 peptide to HeLa cells. Confocal microscopy showed that GFP-L2(108–126) bound to the surface of a HeLa cell, a cell line derived from HPV18-positive cervical cancer. HeLa cells cultured in suspension were allowed to react with purified GFP or GFP-L2(108–126) at 4°C for 1 h. After washing of the cells, fluorescence of GFP bound to the cells was scanned cross-sectionally in a confocal microscope (Fig. 3). HeLa cells incubated with GFP-L2(108–126) showed strong fluorescence around the surface, which was detected as a ring (Fig. 3B), whereas those incubated with GFP did not.

After subsequent incubation of HeLa cells at 37°C for 4 h, the fluorescence of GFP-L2(108–126) was detected by microscopy in the cytoplasms of HeLa cells cultured in suspension (Fig. 3C). Migration of GFP-L2(108–126) from the surface to the perinuclear region was observed more clearly with HeLa cells cultured on a glass slide (Fig. 3E and F). When cells were scanned in the microscope, fluorescent dots were seen probably over the flat surfaces of cells, and after incubation at 37°C, fluorescent masses were seen around the nuclei. The results suggest that the L2 region binds to a cellular protein displayed on the cell surface and that the complex moves toward the inside of the cell.

Quantitative analysis by fluorescence-activated cell sorting with a standard fluorescein isothiocyanate filter indicated that the level of fluorescence of HeLa cells incubated with GFP-L2(108–126) at 4°C for 1 h and washed extensively was much higher than that of HeLa cells incubated with plain GFP (Fig. 4). GFP-L2(108–126) did not bind to HeLa cells that had been treated with 0.2% trypsin at room temperature for 5 min (Fig. 4), indicating that a cell surface protein must be involved in binding.

GFP-L2(108–126) was found to bind not only to HeLa and COS-1 cells but also to some human and rodent cell lines. Because the intensity of the fluorescence of cells that were allowed to react with GFP-L2(108–126) varied from cell line to cell line in preliminary experiments by confocal microscopy, the intensity was measured by flow cytometry and presented as relative to that of the fluorescence of HeLa cells (Fig. 5). The cells that had been cultured on plates were dispersed with PBS containing EDTA (2.5 mM), suspended in fresh medium containing the GFP peptide at 4°C for 1 h, and washed with plain medium before measurement. The human cervical cancer cell lines SiHa and CaSki bound GFP-L2(108–126) at a high level, similar to the level of binding to HeLa cells, but the rodent lymphocyte PAI cell line and insect Sf9 cell line bound almost none of the peptide. COS-1 cells bound the fluorescent peptide in an amount comparable with those bound by the cervical cancer cell lines. The levels of peptide that bound to other cell lines, the human epithelial cell-like cell line 293 (transformed by adenovirus E1), the mouse cell lines C127 (derived from

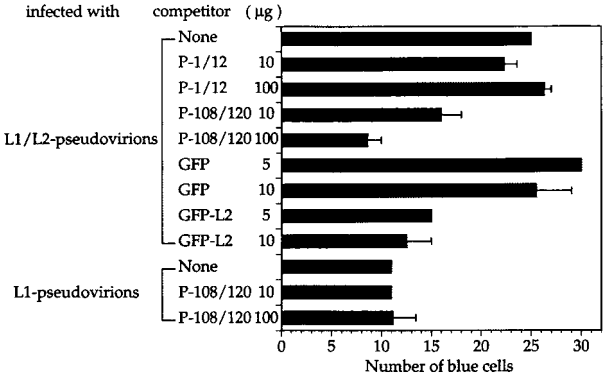

FIG 2. Susceptibility of COS-1 cells preincubated with the L2 peptide to infection with pseudovirions. COS-1 cells (4×10^5) were incubated in PBS (80 μl) containing the L2 peptide P-108/120, P-1/12, GFP-L2(108–126), or GFP for 1 h prior to infection with L1-L2 or L1 pseudovirions. The number of blue cells expressing β-galactosidase was counted 48 h after infection with pseudovirions. Results of three independent experiments are presented with standard deviations (T bars).

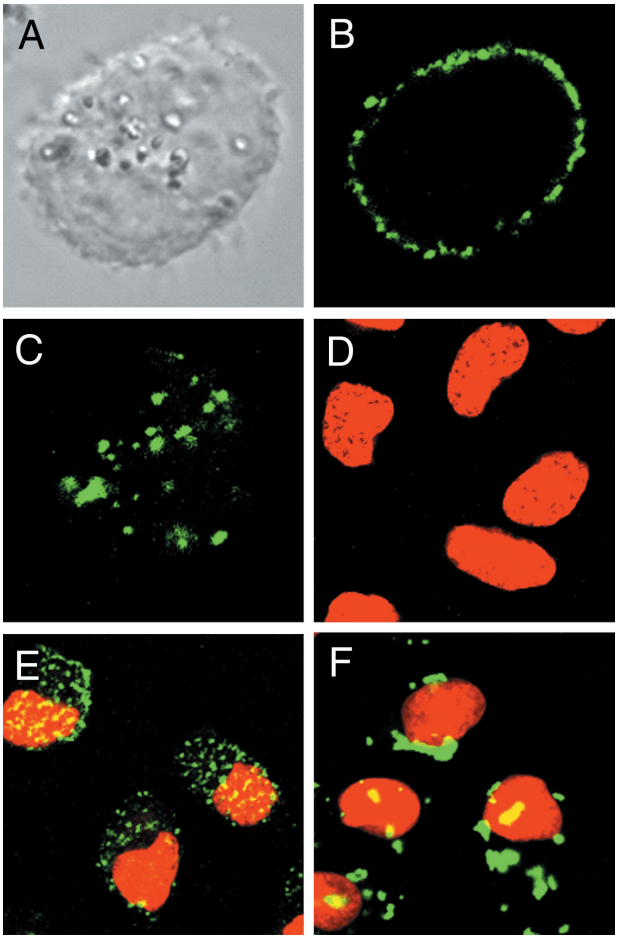

FIG 3. Detection of GFP-L2 fusion protein bound to HeLa cells by fluorescence. (A) Phase-contrast microscopy of a HeLa cell cultured in a spinner flask. HeLa cells cultured in a spinner flask were incubated with GFP-L2(108–126) at 4°C for 1 h (B) and with GFP-L2(108–126) at 4°C for 1 h and at 37°C for 4 h (C). (B and C) The section near the center of a round cell cultured in suspension is presented. HeLa cells cultured on a glass slide were incubated with plain GFP at 4°C for 1 h (D), with GFP-L2(108–126) at 4°C for 1 h (E), and with GFP-L2(108–126) at 4°C for 1 h and at 37°C for 4 h (F). (E) The section near the surfaces of flat cells showed fluorescent dots with parts of nuclei. (F) The section near the centers of cells showed cytoplasmic fluorescence. Fluorescence was examined with a Carl Zeiss LSM510 laser scanning confocal system. (D, E, and F) Nuclei were stained with propidium iodide.

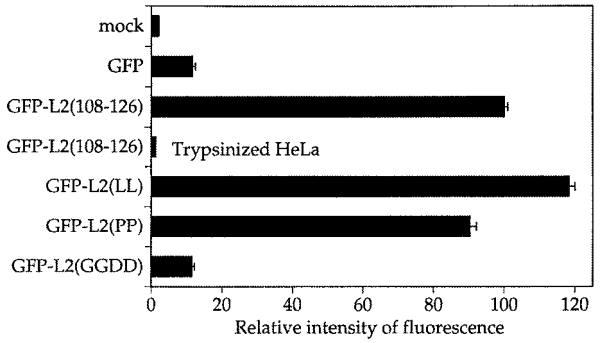

FIG 4. Binding of GFP-L2 fusion protein to HeLa cells. HeLa cells, cultured in suspension, were incubated with various GFP-L2 fusion proteins at 4°C for 1 h and washed, and then the intensity of fluorescence of more than 2×10^5 cells was measured with a FACSCalibur (Becton Dickinson Immunocytometry Systems, Inc.). Relative intensities of fluorescence to HeLa cells incubated with GFP-L2(108–126) are shown with standard deviations (T bars) from three independent experiments. The result with HeLa cells not incubated with GFP is indicated as "mock." "Trypsinized HeLa" indicates cells that had been treated with 0.2% trypsin at room temperature for 5 min before incubation with GFP-L2(108–126).

mouse mammary tumor) and NIH 3T3 (fibroblasts), the rat 3Y1 cell line (fibroblasts), and the human liver cancer lines Alexander and Hep G2, fell between the two groups with the strongest and weakest fluorescence. As can be seen in Fig. 5, the cervical cancer cells bound more GFP-L2(108–126) than the liver cancer cells did, and the human cells appeared to bind more L2(108–126) peptide than the rodent cells did. Among rodent cells, C127 cells, which are susceptible to HPV pseudovirions infection (19), bound more L2 peptide than NIH 3T3 and 3Y1 cells did. Thus, it seems likely that the cells from epithelial tissues have more target proteins for L2(108–126) on their surfaces than those from other tissues.

Mutational analysis of the HPV16 L2 region and infectivity of pseudovirions containing the mutant L2 peptide unable to bind to cell surfaces. Analyses of HPV16 L2(108–126) showed that LVEE at aa 108 to 111 was essential for L2 to bind to the cell surface. Since aa 108 to 111 (LVEE) and aa 118 to 120 (GAP) are highly conserved in mucosal HPVs (10), mutants with substitutions for these amino acids were produced as fusion proteins with GFP (Fig. 1) and examined for their capacity to bind to the surfaces of HeLa cells by flow cytometry (Fig. 4). While the two mutants having LL and PP substituting for GA (aa 118 and 119) bound to the cells at a level similar to that shown with GFP-L2(108–126), the mutant with GGDD substituting for LVEE completely lost the ability to bind. To link the binding assay of the peptide and the infectivity of virions, the pseudovirions having the amino acid substitution

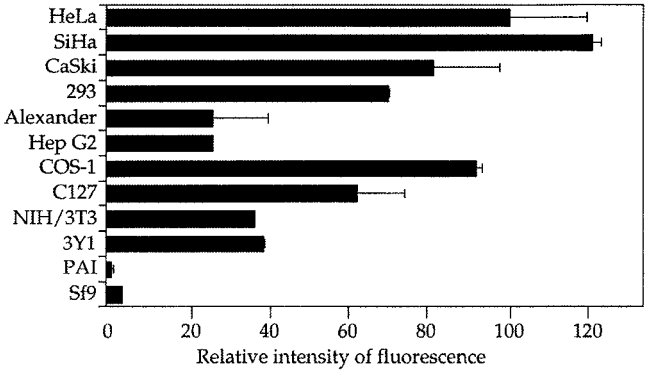

FIG 5. Binding of GFP-L2(108–126) to various cell lines. The intensity of fluorescence of more than 2×10^5 cells incubated with GFP-L2(108–126) was measured with a FACSCalibur (Becton Dickinson Immunocytometry Systems, Inc.). Cells were dispersed with PBS containing EDTA. The average level of intensity specific to GFP-L2(108–126) was calculated by subtracting the mean obtained by incubation with plain GFP from that with GFP-L2(108–126). Intensities of fluorescence relative to that of HeLa cells are presented with standard deviations (T bars) from three independent experiments.

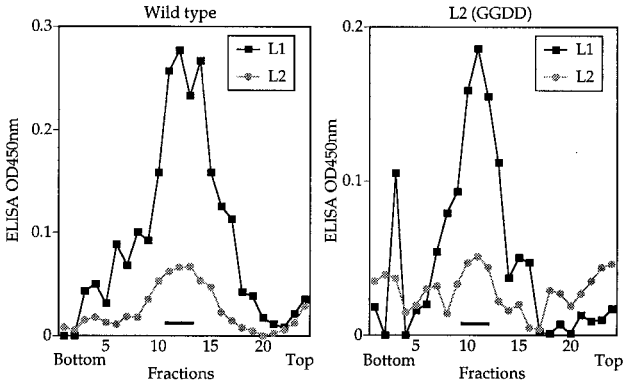

FIG 6. Sucrose gradient sedimentation of HPV16 pseudovirions. Pseudovirions containing wild-type or mutant L2 with the amino acid substitution of GGDD for LVEE, purified by CsCl equilibrium density gradient centrifugation, were sedimented in a sucrose gradient (10 to 60% [wt/vol] in PBS) by centrifugation and fractionated (0.2 ml/fraction) from the bottom. L1 and L2 in each fraction were denatured in carbonate buffer (pH 9.6) and measured by enzyme-linked immunosorbent assay (ELISA) using anti-L1 and anti-L2 antibodies. Pools of fractions indicated by bars contained approximately 4.5×10^3 copies of DNase I-resistant indicator plasmid per ng of L1 protein. OD450nm, optical density at 450 nm.

were constructed and examined for their infectivity in COS-1 cells.

Mutant L2 with substitution of GGDD for LVEE was packaged into pseudovirions constructed in vitro. Capsids generated in Sf9 cells infected with a recombinant baculovirus expressing L1 and the mutated L2 were extracted and purified as described previously (8, 9). Then mutant pseudovirions were constructed from the disassembled mutant capsids and reporter plasmid DNA in vitro and purified by CsCl centrifugation as described previously (9). The fractions with a density of 1.31 g/ml, which contained icosahedral particles as seen by electron microscopy (data not presented) and plasmid DNA resistant to DNase I (data not presented), were sedimented in a sucrose gradient (10 to 60%) by centrifugation and fractionated. The presence of L1 and L2 in each fraction was examined by enzyme-linked immunosorbent assay using anti-L1 and anti-L2 antibodies (8, 9) (Fig. 6). Clearly, L1 and L2 were copurified in fractions 10 to 12. The amounts of L2 in relation to L1 in these fractions were comparable to those of wild-type pseudovirions (8, 9). The data indicate that mutated L2 was incorporated into the capsids and that the substitution of GGDD for LVEE did not affect the production of pseudovirions.

The mutant pseudovirions [L1-L2(GGDD)] were found to be less infectious than the wild type (L1-L2) (Table 1). For the infectivity assay, COS-1 cells were infected, as previously described (9), with freshly constructed and purified pseudovirions. It should be noted that COS-1 cells, whose parental cell line, CV-1, can bind with L1 capsids (5), were positive for binding with GFP-L2(108–126) (Fig. 5). Input virions were adjusted to the L1 content estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by silver staining. One nanogram of virions, wild-type or mutant, contained approximately 4.5×10^3 copies of the DNase I-resistant reporter plasmid. Indirect immunofluorescence staining using anti-HPV16 L1 antibody (PharMingen, San Diego, Calif.) showed that wild-type and mutant pseudovirions used in this assay bound equally to the cell surface (data not presented). The number of blue cells produced in COS-1 culture infected with the mutant pseudovirions dropped to a level similar to that obtained by infection with L1 pseudovirions (Table 1). Thus, the mutant pseudovirions were less infectious than the wild-type pseudovirions due to the loss of the L2 effect, which strengthens the infectivity of the pseudovirions (9, 24). It was concluded from these results, along with the data in Fig. 2, that the binding of the L2 region that contains the common neutralization epitope with a surface cellular protein is necessary for efficient HPV infection.

DISCUSSION

In this study a competition assay showed that the peptide with the N-terminal sequence of HPV16 L2(108–120) interfered with the infectivity of pseudovirions in COS cells, and confocal microscopy showed that the L2(108–126) peptide tagged with GFP attached to the HeLa cell surface at a low temperature and entered the cytoplasm when the temperature was raised to 37°C. The competition assay suggests the presence of a cell surface receptor for the region from aa 108 to 120 of L2, to which the GFP peptide can bind. The binding and visualized behavior of the L2 peptide (Fig. 3) are considered to reflect the behavior of L2 in the virion, because the amino acid substitutions that prevented the peptide from binding were found to lower the infectivity of the pseudovirion having the same mutations. Thus, the results obtained in this study suggest that L2(108–126), displayed on the surfaces of capsids (8), is involved in an early step of HPV infection by binding with the cellular receptor at the time of the attachment of virions to cells.

However, binding of L2 to the cell receptor may be unnecessary for adsorption because the capsids without L2 are believed to attach to cells as efficiently as the capsids with L2 (20, 25). It is possible that L2 binding may be a passive reaction at adsorption, which is mediated mainly by L1. On the other hand, it is clear that L2 has a certain role in enhancing infectivity in pseudovirion assays (Fig. 2) (18, 24). Perhaps L2 is active at a postadsorption step: at the entry of virions into cells or otherwise at a later step before expression of the viral genes (18).

The cell surface receptor for L2 appears to be different from those for L1, as shown by the competition experiments in which the L2 peptide did not interfere with the infectivity of L1 pseudovirions (Fig. 2). The L1 pseudovirions are less than half as infectious as the L1-L2 pseudovirions (24) (Fig. 2) and so are the pseudovirions with the substitution mutation (Table 1).

TABLE 1. Infectivities of pseudovirions in COS-1 cells

Capsid(s) of pseudovirions	No. of blue cells[a]/100 ng of L1				
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
L1-L2	20	17	25	22	25
L1-L2(GGDD)	5	4	9	9	8
L1	ND[b]	ND	ND	10	ND

[a] Blue cells represent those expressing β-galactosidase as a result of HPV16 pseudovirion infection.
[b] ND, not determined.

Furthermore, saturation of the surface L2 receptors with the L2 peptide lowered the measured level of infectivity of the L1-L2 pseudovirions to that of L1 pseudovirions (Fig. 2). Thus, binding to L1 and L2 receptors seems to enhance infectivity, compared with binding to L1 receptors alone. Possibly, binding to both receptors may help in efficient internalization of the virions.

Cellular surface protein binding with L2 may determine part of the cell tropism of mucosal HPVs. The putative receptor for L1 has been reported to be a protein widely expressed and evolutionarily conserved among cells derived from a variety of tissues (17, 20, 25). For example, α_6 integrin, a probable candidate for the receptor for HPV6b (5, 15) but not the obligatory receptor for BPV4 (23), is a molecule expressed widely on the surfaces of cells derived from various tissues. Thus, binding of L1 alone to the putative receptor does not account for the tropism of mucosal HPVs. Since cellular protein binding with HPV16 L2 seems to be expressed more abundantly in epithelial cells, especially cells derived from cervical cancers, than those of other origins (Fig. 5), and since the amino acid sequence of the HPV16 L2 region is conserved in mucosal HPVs, it is possible that conditions in cells expressing the target protein for L2 are favorable for mucosal HPVs to infect.

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