Human Papillomavirus Type 6b Virus-Like Particles Are Able To Activate the Ras-MAP Kinase Pathway and Induce Cell Proliferation

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Papillomaviruses are nonenveloped double-stranded DNA tumor viruses that cause a range of proliferative lesions upon infection of epithelial cells (8). These viruses are the causative agent of warts (plantar, laryngopharyngeal, and genital) (2) and the critical factor in the formation of anogenital cancer (30). The first step in viral infection is the binding of the virus to its specific receptor upon a host cell. Due to the lack of in vitro replication systems, many steps in the papillomavirus life cycle have not been elucidated; however, the advent of virus-like particle (VLP) technology is beginning to overcome this problem. Using VLPs, we have recently identified the α6 integrin as a papillomavirus receptor (7, 18). Expression of α6 integrin in a receptor-negative cell line confers the ability to bind virus, indicating that α6, paired with either the β1 or β4 integrin, is both necessary and sufficient for papillomavirus binding (18). The α6β4 integrin is expressed in the basal layers of stratified squamous epithelium (12), a distribution that matches the site of productive papillomavirus infection.

The α6β4 complex is an integral part of the hemidesmosome complex and, as a receptor for laminins 1, 2, 4, and 5, is involved in the attachment of epithelial cells with the basement membrane (24). α6β4 differs from all other integrins in that the β4 subunit has a long cytoplasmic tail of 1,000 amino acids that is structurally different from other beta subunits. Recent reports have shown that the ligation of many integrins causes receptor activation and/or clustering, which results in intracellular signaling events that influence cell proliferation. For example, tyrosine residues in β4 are phosphorylated in response to α6β4 receptor ligation by laminin, resulting in activation of the Ras-MAP kinase pathway, phosphatidylinositol 3-kinase, and the stimulation of cell growth (15–17). Conversely, expression of β4 integrin in a rectal carcinoma cell line (RKO) has been reported to result in G1 growth arrest, activation of p21, and apoptosis (4). This has led to the suggestion that integrins give spatial clues to cells and indicate appropriate responses, such as growth, differentiation, or apoptosis. Thus, in the skin, keratinocytes in contact with the basement membrane have activated α6β4, which promotes cell growth via the Ras-MAP kinase pathway, while keratinocytes lost from the basement membrane lose this signal and differentiate.

Signaling pathways determine a cell’s ability to respond to external stimuli via the induction of transcription factors. There is mounting evidence that virus-receptor interactions are not merely conduits of viral entry to the cell but that viruses may utilize signaling pathways, via these receptors, to induce a cellular state that is more receptive for infection. For example, simian virus 40 (SV40) rapidly and transiently induces expression of the c-myc, c-sis, and c-jun genes upon ligation of its receptor, the major histocompatibility complex class I receptor, causing the cell to proliferate (5). Given that the α6β4 integrin is able to signal cells via the Ras-MAP kinase pathway to induce cell growth and that the α6 integrin is a receptor for this virus, we wondered if papillomavirus might also transduce a signal to cells upon α6β4 ligation and what the nature of such a signal might be.
In this work, we provide detailed evidence that exposure of epithelial cells to papillomavirus VLPs (PV-VLPs) results in cell proliferation in a dose-dependent manner. This proliferation is dependent upon VLPs having a correct conformation and the MAP kinase kinase MEK1, suggesting that the Ras-MAP kinase pathway is activated upon VLP binding. Indeed, VLP binding to cells results in phosphorylation of the β4 integrin and activation of the Ras-MAP kinase pathway. Virus exposure to cells causes the recruitment of the adapter protein Shc to β4 and results in the activation of Ras, Raf, and Erk2 and the upregulation of c-myc mRNA.

MATERIALS AND METHODS

Cells, VLPs, and antibodies. Human papillomavirus type 6b (HPV6b) L1 VLPs were produced in Sf9 cells by using HPV6b L1 recombinant baculovirus and purified as previously described (20). All VLP batches were checked for purity and conformation by electron microscopy, enzyme-linked immunosorbent assay with a panel of conformational and nonconformational antibodies, and Western blotting. A431 cells were obtained from the American Type Culture Collection. The following antibodies were used: anti-α6 (GoH3; Serotec, London, England); anti-HPV6b L1 and anti-HPV6b L2 (a gift from Wen-Jun Liu, University of Queensland, Brisbane, Australia); and anti-β4 (3E1; Gibco-BRL).

Cell proliferation assay. Cell proliferation was measured using the cell proliferation kit from Amersham-Pharmacia (Sydney, Australia). Human A431 epithelial cells were seeded in eight-well chamber slides (20,000 cells/well) and incubated overnight in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal calf serum (FCS) to 10%. Cells were washed, DMEM minus FCS was added, and incubation was continued for 2 days. Cells were treated with DMEM supplemented with FCS to 10% or HPV6b L1 VLPs (0.16 to 8 ng/ml) in DMEM for 16 to 48 h at 37°C. Labeling reagent, containing 5-bromo-2’-deoxyuridine (BrdU) and 5-fluoro-2’-deoxyuridine at a 1:10 molar ratio, was added for the final 2 h of the incubation, after which the cells were briefly washed in phosphate-buffered saline (PBS) and fixed in acid-ethanol (90% ethanol, 5% glacial acetic acid, 5% water) for 30 min. Fixed cells were washed three times with PBS before incubation with an anti-BrdU monoclonal antibody (1:100) plus DNase for 60 min at room temperature. Following a further three washes in PBS, cells were exposed to goat anti-mouse immunoglobulin (Ig)-horseradish peroxidase (HRP) conjugate (1:100) for 30 min at room temperature. Finally, BrdU-containing cells were visualized by incubation in a 25-μg/ml diaminobenzidine (DAB) solution for 10 min before the cells were washed in water, counterstained with hematoxylin, and mounted. BrdU-positive cells from five random fields were counted under light microscopy. VLP treatments were as follows. To denature VLPs, 1.6 ng was incubated at 100°C in PBS for 20 min. For monoclonal antibody blocking, 1.6 ng of VLPs was incubated for 30 min with monoclonal antibodies against HPV6b L1 or L2 (5 μl of ascites fluid) in a volume of 1 ml of PBS. For cell pretreatment, cells were either incubated with 5 μl of an anti-α6 integrin (GoH3) in 400 μl of DMEM at 37°C for 30 min or treated with the indicated concentrations of PB908059 (NEB, Beverly, Mass.) for 60 min prior to VLP addition.

Tyrosine phosphorylation assays. (i) Total cell phosphotyrosine. A431 cells (2 × 10⁶) were serum starved for 48 h before being washed three times with PBS, and VLPs were added (0 to 2.25 mg/ml). After 20 min, the cells were washed with PBS containing 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EGTA and lysed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 2 mM EDTA plus 1 mM orthovanadate, 1 mM PMSF and 1 mM EGTA) for 5 min at 4°C. The protein concentrations were determined by the Bio-Rad BCA assay and equalized with lysis buffer. Lysate samples (5 μl) were spotted in triplicate onto nitrocellulose and allowed to air dry. Total phosphotyrosine was detected using a polyclonal antiphosphotyrosine antibody (Upstate Biotechnology).

(ii) β4 phosphorylation. A431 cells (subconfluent, 80-cm² flask) were serum starved overnight before being washed three times with PBS and treated with PBS-EDTA (0.05%) for 10 min. Cells were scraped into serum-free DMEM, placed into new flasks, and incubated overnight at 37°C. The cells were washed with PBS before being treated with VLPs (1 μg/ml) and 10% fetal calf serum (FCS) was added, and incubation was continued for 2 days. Cells were treated with EGF (250 ng/ml) or EGF (100 ng/ml) at 37°C in serum-free DMEM. The cells were washed once with medium before being scraped into lysis buffer (50 mM HEPES [pH 7.4], 1 mg of bovine serum albumin [BSA] per ml) and then incubated in phosphate-free DMEM plus additives containing 10 mM HEPES (pH 7.4), 0.9% NaCl, 1 mM EGTA, 1 mM PMSF, 1 mM sodium vanadate, 1 mM sodium fluoride, plus 1 μg of aprotinin and 1 μg of leupeptin per ml). The protein concentration was determined using the Bio-Rad protein assay dye reagent concentrate.

Equal amounts of each cell extract were immunoprecipitated overnight at 4°C with β4 integrin antibody (3E1). Protein G-Sepharose was added, and the tubes were mixed for a further 2 h before the immunocomplexes were washed three times with cold 1× PBS. Loading buffer and β-mercaptoethanol were added to each of the samples, which were then boiled before undergoing SDS-PAGE and subsequent transfer to Hybond C. The blots were blocked for 20 min using 3% skim milk powder in 1× PBS and then incubated overnight with an anti-Shc antibody (Upstate Biotechnology). After being washed in water, the blots were incubated for 1 h with an anti-rabbit IgG-HRP-conjugated secondary antibody. The blots were then washed using water and 1× PBS plus 0.05% Tween 20 before the Shc bands were detected using chemiluminescence.

Ras-GTP loading assay. A431 cells in 10-cm plates were transfected with 10 μg of H-Ras-expressing plasmid DNA using Lipofectamine and incubated for 36 h before being serum starved in DMEM for 18 to 24 h. Cells were washed in phosphate-free DMEM containing additives (10 mM HEPES [pH 7.4], 1 mg of bovine serum albumin [BSA] per ml) and then incubated in phosphate-free DMEM plus additives containing 100 to 150 μM of [³²P]lithophosphate per ml for 4 h at 37°C. After this incubation period, the cells were placed on ice and washed with cold phosphate-free DMEM. Cells were then treated with VLPs (360 ng/ml) or EGF (100 ng/ml) at 37°C in serum-free DMEM. The cells were washed once with medium before being scraped into lysis buffer (50 mM HEPES [pH 7.4], 1% Triton X-100, 100 mM NaCl, 5 mM MgCl₂, 10 mM benzamidine plus 1 mg of BSA, 10 μg of leupeptin, 10 μg of aprotinin, and 10 μg of soybean trypsin inhibitor per ml) and transferred into microcentrifuge tubes, and the cell debris was removed by centrifugation. The supernatant was preincubated by rotation at 4°C for 15 min using a rabbit anti-rat IgG-protein G-Sepharose bead slurry, with the addition of 0.5 M NaCl, 0.5% deoxycholate, and 0.05% SDS. These beads were pelleted, and the supernatant was then added to a tube containing rabbit anti-rat IgG-protein A-Sepharose Y13-259 (anti-Ras antibody) bead slurry and rotated at 4°C for 40 min before the beads were washed eight times with wash buffer (50 mM HEPES [pH 7.4], 500 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, 0.005% SDS). After the final wash, proteins were eluted from the beads by incubating at 68°C for 20 min in 2 mM EDTA, 2 mM dithiothreitol (DTT), 0.2% SDS, 0.5 mM GTP-0.5 mM GTP. These samples were then loaded on polyethylenimine-cellulose plates (Merck) and run in 1 M LiCl. After being dried, the plate was visualized using a phosphorimager, and the GDP and GTP spots were quantitated by densitometer analysis.

Assay for Raf activity. (i) Cell treatments and membrane preparation. A431 cells in T150 flasks were serum starved in DMEM for 18 to 24 h before being washed in serum-free DMEM and treated with FCS (10%) or VLPs (360 ng/ml of medium) at 37°C for various incubation times. Following treatment, the cells were then washed twice with PBS before being scraped off and collected in buffer A (10 mM Tris [pH 7.4], 25 mM NaF, 5 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 200 μM sodium vanadate, plus 2 μg of aprotinin and 3 μg of leupeptin per ml). The samples were kept on ice for 10 min and then homogenized in a Dounce homogenizer with 50 strokes. The homogenate and the supernatant were spun in an ultracentrifuge at 120,000 × g for 30 min at 4°C to isolate the cytosol (S100) and membrane (P100) fractions. The pellet (P100) was washed and then resuspended in buffer A. The protein concentration of the P100 fraction was determined using the Bio-Rad protein assay before the samples were snap-frozen in aliquots and stored at −70°C until analysis using the Raf activation assay or epidermal growth factor receptor assay was performed.

(ii) Raf activation assay. The Raf activity assay was done by the method of Roy et al. (16).

MAP kinase assay. A431 cells (10°) were serum starved in DMEM for 48 h before being washed in serum-free DMEM and treated with FCS (10%) or VLPs.
(300 ng, 1:1 receptor-virus ratio). Following treatment, cells were washed three times with PBS, lysed in 100 μl of SDS-PAGE sample buffer (62.5 mM Tris [pH 6.8], 2% SDS, 10% glycerol, 0.1% bromophenol blue, 50 mM DTT), and collected into microcentrifuge tubes. Samples were sonicated for 15 s, 2-mercaptopethanol was added to a final concentration of 10%, samples were boiled for 5 min and centrifuged for 5 min at 14,000 × g, and 20 μl was loaded onto an SDS-polyacrylamide gel. Following electrophoresis, the proteins were transferred to nitrocellulose membrane at 100 V for 60 min. The resulting blot was blocked in 5% skim milk–TBST (20 mM Tris, 137 mM NaCl, and 0.05% Tween 20) for 60 min, washed three times in TBST, and incubated with primary antibody, either anti-MAP kinase (New England Biolabs 91015) or anti-phospho-MAP kinase (New England Biolabs 9102) or anti-laminin (Sigma) diluted 1:2,000 in TBST, for 60 min. Blots were washed four times, and proteins were detected by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

**RESULTS**

**VLP binding results in cell proliferation.** The ligation of the α6β4 integrin by laminin has previously been shown to result in the induction of cell proliferation in cells via the Ras-MAP kinase pathway (15–17, 22). Given that we have shown that there is a direct interaction between the α6 integrin and papillomavirus VLPs (7, 18), we decided to investigate whether the act of virus attachment to cells would also activate cell proliferation and drive resting epithelial cells into the cell cycle. Serum-starved human A431 epithelial cells (2 × 10⁶) were exposed to HPV6b L1 VLPs (from 0.16 to 8 ng) for 16 h, including a pulse-label with BrdU for the final 2 h. FCS-supplemented DMEM was used as a positive control. BrdU-positive nuclei were detected using a monoclonal antibody and visualized with DAB, and five random fields were counted (Fig. 1). It was observed that cell proliferation was induced in VLP-treated cells in a dose-dependent manner, with 1.6 and 8 ng of VLPs giving the same stimulation level as 10% FCS. The level of proliferation induced by FCS and the two highest VLP doses was significantly increased compared to controls (P < 0.001).

To ensure that the proliferation observed was due to the VLPs themselves, VLPs were incubated with monoclonal antibodies against the HPV6b L1 protein or L2 protein (as a control) for 30 min prior to exposure to cells (Fig. 1B). It can be seen (Fig. 1B) that treatment with anti-L1 resulted in the loss of cell proliferation, while anti-L2 treatment had no effect. As these VLPs contain only L1 protein, this suggests that it is the interaction of L1 with cells that caused cellular proliferation. Moreover, L1 must be present in the form of VLPs, as the use of the α6 ligand laminin, were not attempted, as these have previously been shown to themselves activate growth.

**VLP binding induces tyrosine phosphorylation in whole-cell extracts and of β4 integrin.** Given that VLPs were able to induce cell proliferation, we were curious as to how this was achieved. As VLPs contain no viral DNA, the normal virus program to control cell growth could not be initiated. There-
fore, we next investigated whether there were any intracellular signaling events caused by papillomavirus binding by examining the total cellular level of tyrosine phosphorylation. A431 cells (2 × 10⁵) were serum starved before being treated with increasing concentrations of PV-VLPs for 20 min. Cell extracts were prepared and dot blotted in triplicate, and total cell tyrosine phosphorylation was measured by immunoblotting with an antiphosphotyrosine antibody (Upstate Biotechnology). (B) β4 integrin is phosphorylated on tyrosine upon PV-VLP treatment. Serum-starved A431 cells were treated with VLPs for 5, 30, or 60 min before the β4 integrin was immunoprecipitated (IP), and phospho-β4 was detected using a polyclonal antiphosphotyrosine (Tyr-P) antibody (Upstate Biotechnology). C, no treatment.

FIG. 2. Human papillomavirus VLPs induce tyrosine phosphorylation events in A431 cells. (A) Total-cell phosphotyrosine. Serum-starved A431 cells were treated with increasing amounts of VLPs for 20 min before total cell protein was extracted, equal amounts were blotted, and total phosphotyrosine was detected using a polyclonal antiphosphotyrosine antibody (Upstate Biotechnology). (B) β4 integrin is phosphorylated on tyrosine upon PV-VLP treatment. Serum-starved A431 cells were treated with VLPs for 5, 30, or 60 min before the β4 integrin was immunoprecipitated (IP), and phospho-β4 was detected using a polyclonal antiphosphotyrosine (Tyr-P) antibody (Upstate Biotechnology). C, no treatment.

FIG. 3. PV-VLP binding causes recruitment of the adapter protein Shc. Following treatment with EGF or PV-VLPs (10, 20, or 30 min), cell extracts were prepared, and β4 was immunoprecipitated (IP) with the anti-β4 integrin antibody 3E11. Immunocomplexes were subjected to SDS-PAGE and Western blotting, and Shc was detected. As a control (C), whole-cell extract (WCE) was immunoblotted to indicate basal Shc levels.

VLP binding causes recruitment of the adapter protein Shc. We next examined whether the PV-VLP-induced activation of β4 was able to initiate recruitment of the adapter protein Shc. Shc is an SH2-phosphotyrosine-binding domain adapter that links tyrosine kinases and other tyrosine-phosphorylated proteins to the Ras-MAP kinase pathway by recruiting protein complexes (mainly Grb2-mSOS) to the plasma membrane. There are three forms of Shc, p66Shc, p52Shc, and p46Shc. A431 cells were serum starved for 18 to 24 h before exposure to VLPs or EGF. β4 integrin was immunoprecipitated from cell lysates before Shc was detected by immunoblotting (Fig 3). It can be seen that PV-VLP treatment results in the time-dependent recruitment of p52⁵Shc and p46⁶Shc to β4 integrin, with maximal binding between 10 and 20 min (Fig. 3). To our knowledge, this is the first example of virus-induced recruitment of Shc to its receptor. p66Shc did not appear to be recruited to the β4 integrin complex even though all three Shc isoforms are present in equal amounts (Fig. 3), a finding consistent with laminin-induced α6β4 activation (16). As expected, EGF did not induce Shc recruitment to β4 integrin.

VLP binding results in activation of Ras and Raf. A predictable consequence of Shc binding to the β4 integrin complex is recruitment of Grb2-mSOS and activation of Ras to its GTP-bound state. Therefore, Ras-GTP loading experiments were performed to examine if PV-VLP-mediated ligation of α6β4 was able to activate Ras. Cells were serum starved and labeled with [³²P]orthophosphate before being treated with PV-VLP or EGF. Ras was immunoprecipitated from cell lysates, and GDP- or GTP-bound Ras was separated by thin-
serum-starved A431 cells were treated with VLPs for various times before being harvested and Western blotted. The detection of phosphorylated Erk was achieved by using a phospho-specific antibody. As a control, cells were stimulated with 10% FCS. It can be seen that the binding of VLPs causes the activation of Erk, with maximal activation observed at 30 min (Fig. 6). Activation was lost by 40 min. Once again, the activation by FCS was more rapid, with maximal phosphorylation at 10 min. The antibodies used in this assay are able to detect both Erk1 and Erk2, but in our A431 cells it appears that Erk2 (p42) was more abundant and activation was mainly of Erk2, while Erk1 (p44) appeared to be phosphorylated to a much lesser extent.

Virus binding induces c-myc expression. A consequence of many signaling pathways, including Ras-MAP, is the activation of transcription factors which in turn lead to transcriptional activation of immediate-early genes. One of the immediate-early genes induced by Ras-MAP pathway activation (via Erk2) is c-myc. Therefore, we investigated the regulation of c-myc in response to virus binding. A431 cells were serum starved before the addition of VLPs, and total cell RNA was extracted and Northern blotted for c-myc. Figure 7 shows that PV-VLP binding induced a threefold increase in c-myc mRNA level by 60 min (Fig. 7).

VLP-mediated proliferation requires the MAP kinase pathway. A consequence of the induction of c-myc via the Ras-MAP pathway would be the activation of cell proliferation, as observed in Fig. 1. Therefore, we wished to know if the observed VLP-mediated cell proliferation was acting via the MAP kinase pathway. Serum-starved A431 cells were treated for 60 min with PB98059, a highly selective inhibitor of the MAP kinase cascade. This compound selectively inhibits the activation and phosphorylation of MEK1 with a 50% inhibitory concentration (IC50) of 5 to 10 μM; it is known to inhibit MEK2, but the IC50 is much higher (50 μM). Following treatment, PV-VLPs (5 μg) were added to cells, and incubation was continued for 16 h, including a pulse-label with BrdU for the

layer chromatography. Within 5 min of PV-VLP treatment, increased amounts of activated, GTP-bound Ras were observed. Elevated levels of Ras-GTP were observed until 20 min, falling away at 30 min (Fig. 4). The level of this activation was not as pronounced as that previously observed for laminin-induced activation but was highly reproducible (15).

To determine whether the level of Ras activity stimulated by PV-VLPs was sufficient to activate Ras-dependent signaling, we next examined if ligation of α6β4 by VLPs resulted in the activation of Raf. Endogenous Raf-1 activity was measured using a well-established sensitive assay (21). Serum-starved cells were treated with VLPs or serum, and the Raf activity associated with plasma membranes was measured in a two-stage assay, with phosphorylation of myelin basic protein as a final readout. It can be seen that the addition of PV-VLPs clearly results in Raf activation, with a 2.13-fold increase in Raf activity after 10 min which is sustained at 20 and 30 min (Fig. 5). This activity fell close to baseline values by 40 min, indicating that the activation by VLPs was rapid but quickly lost. FCS (10% in DMEM) was used as a positive control and gave a fourfold increase in Raf-1 activity after 5 min.

Erk is phosphorylated in response to virus binding. We next examined if ligation of α6β4 by VLPs resulted in activation of the MAP kinase Erk. Erk is downstream of the Ras/Raf pathway and is phosphorylated by the MAP kinase kinase MEK. Serum-starved A431 cells were treated with VLPs for various times before being harvested and Western blotted. The detection of phosphorylated Erk was achieved by using a phosphorylation-specific antibody. As a control, cells were stimulated with 10% FCS. It can be seen that the binding of VLPs causes the activation of Erk, with maximal activation observed at 30 min (Fig. 6). Activation was lost by 40 min. Once again, the activation by FCS was more rapid, with maximal phosphorylation at 10 min. The antibodies used in this assay are able to detect both Erk1 and Erk2, but in our A431 cells it appears that Erk2 (p42) was more abundant and activation was mainly of Erk2, while Erk1 (p44) appeared to be phosphorylated to a much lesser extent.

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final 2 h. BrdU-positive nuclei were detected as mentioned above, and five random fields were counted (Fig. 8). As previously observed, PV-VLPs induced cell proliferation, as evidenced by the increase in BrdU-positive nuclei. The addition of PB98059 resulted in a dose-dependent decrease in VLP-mediated cell proliferation. The IC50 was 4.04 μM, suggesting that this is inhibition of MEK1 and not MEK2. These results indicate that VLP-mediated cell proliferation requires a functional Ras-MAP kinase pathway to induce cell proliferation.

**FIG. 7.** Induction of c-myc by PV-VLP binding. A431 cells were serum starved before treatment with EGF or VLPs for 20, 30, or 60 min. Total RNA was extracted and Northern blotted, and the blots were probed for human c-myc. Control, no treatment.

**FIG. 8.** A431 cells were serum starved for 2 days and then treated with the indicated amounts of the MEK1 inhibitor PB98059 for 60 min before the addition of 5 μg of VLPs for 16 h. BrdU was added for the final 2 h and, following cell fixation, detected using anti-BrdU antibodies and DAB staining (see Materials and Methods). BrdU-positive cells in five random fields were counted. Control, no treatment.

**DISCUSSION**

The ability of viruses to activate cell signaling events upon engagement of their cellular receptor has recently become apparent, but the exact nature of these signaling events has not been clear. The main finding presented here is that HPV6b L1 VLPs are able to induce growth in resting cells via coordinated stimulation of the Ras-MAP kinase pathway, the first demonstration of a virus specifically activating this pathway. While we have not shown here that this activation occurs directly via α6β4 integrin, our findings are consistent with that hypothesis. Maximal activation of cell proliferation was achieved using 1.6 ng of VLPs on approximately 40,000 cells. Hypothetically, this represents about two virus particles per receptor, in that we have previously shown there are 10^4 VLP-binding sites per cell, using CV1 cells (20). The exact number of receptors on A431 cells is not known, although cell sorting analysis of α6β4 expression shows approximately the same number as in CV1 cells. Other viruses have previously been shown to activate cell signaling pathways. For example, SV40 has been shown to activate primary response genes via a protein kinase C-dependent pathway (5), while human immunodeficiency virus (HIV) rapidly induces tyrosine phosphorylation of the protein tyrosine kinase Pyk2 upon binding to its chemokine coreceptor, CXCR4 or CCR5 (6, 28). Epstein-Barr virus, which binds to CD21, is able to activate resting B cells via a signaling pathway involving NF-κB (26). These signaling events appear to be important for virus replication, as their blockade is able to inhibit virus entry. For example, blockage of SV40 signaling by Geneticin did not affect virus binding, but virus uptake was severely reduced (5). Similarly, HIV entry was inhibited by the blockade of signaling from CCR5 and CXCR4 using pertussis holotoxin (1).

The activation of Ras via the α6β4 integrin has previously been shown to occur upon the engagement of its normal ligand, laminin, as well as by anti-β4 antibodies when attached to 2.5-μm beads (15). These means gave 25 to 33% activation of Ras over a 60-min time period, a more pronounced and sustained activation than that induced by papillomavirus particles. There are two possible explanations for the lower level of Ras activation induced by PV-VLPs. First, PV-VLPs are rapidly internalized, usually within 30 min (29), which may not allow such a sustained signal as would occur with plastic-bound laminin or antibodies bound to beads. Second, antibody can activate α6β4 when coupled to beads (15) because activation is thought to require dimerization or oligomerization of the integrin. It is possible that while virus particles 50 nm in diameter can certainly cause integrin cross-linking, this is achieved less efficiently than with 2.5-μm antibody-coated beads.

The PV-VLP-mediated phosphorylation of β4 was rapid (5 min) before dropping to low levels at 30 min. This is in agreement with previous data showing that maximal phosphorylation of β4 by laminin occurs at 2 min and by β4-antibody cross-linking at 10 min (16). Like many cytokine receptors, α6β4 lacks an intracellular catalytic domain and therefore must rely on an association with a cytoplasmic tyrosine kinase. The tyrosine phosphorylation of β4 most likely occurs via an integrin-associated kinase, but the identity of this kinase awaits elucidation. It should be noted that in A431 cells, α6 associates...
only with β4, and no α6β1 is present, so the downstream pathways from the β1 integrin were not investigated.

The activation of α6β4 is central to the control of keratinocyte proliferation, which explains why loss of cells from the basement membrane results in the onset of differentiation. This control appears to be mediated by the adapter protein Shc (15). Our data indicate that the β4 integrin is activated by PV-VLP binding, and this induces the recruitment of p52Shc and p46Shc to β4, although it is not known if this association is a direct interaction. The activation of β4 by laminin also results in tyrosine phosphorylation of β4, which in turn results in the specific recruitment of p52Shc, with a minor amount of p46Shc present (16). Although all three Shc isoforms are present equally in A431 cells (Fig. 2), PV-VLP-mediated activation of β4 resulted in the equal association of p52 and p46Shc, which is different from the response observed for laminin-mediated activation. In both cases, p66Shc, a negative regulator of Shc activity, was absent. EGF treatment did not result in Shc recruitment to β4 integrin, as expected.

The binding of VLPs resulted in selective activation of Erk2. Erk2 activation has been shown to specifically activate c-myc, whereas Erk1 activation led to activation of the transcription factor Elk-1 (3). Consistent with our data, activation of Erk2 was also observed for laminin-5-stimulated α6β1 integrin (27). Treatment with VLPs gave a time-dependent increase in the level of c-myc mRNA (Fig. 6) and entry into the cell cycle. The presence and activation of α6β4 appears to be required for cell cycle entry, as transgenic mice carrying deletions in the cytoplasmic tail of the β4 integrin display proliferation defects (19).

An interesting question raised by this study is why papillomavirus would rapidly and transiently activate the Ras-MAP kinase pathway. One possible explanation is that such activation and induction of growth would be advantageous to viral replication. Indeed, many viruses, such as vaccinia virus, SV40, HIV-1, herpesvirus, and coxsackievirus, depend on the activated Ras-MAP kinase pathway for growth (9–11, 13, 23, 25). While it is not known if papillomavirus requires a dividing cell in which to initiate replication, due to the limitations of current replication systems, it is known that initial viral replication is only observed to occur in basal keratinocytes, and this is the site of the only cells undergoing cell division in the skin epidermis. Indeed, basal keratinocytes have been shown to exist as either quiescent stem cells or rapidly dividing “transient amplifying” cells (14). The transient amplifying cells are pushed into the suprabasal layer as they divide, resulting in the loss of interaction between α6β4 and laminin, causing deprivation of this positive growth signal and subsequent exit from the cell cycle. We have shown previously that basal and suprabasal cells both express the α6 integrin and bind papillomavirus (7). We therefore postulate that the ability of papillomavirus to signal cells via Ras may cause resting stem and suprabasal cells to proliferate and thus allow the virus to initiate replication. This would result in an increased pool of cells potentially able to be infected by papillomavirus. Unfortunately, this hypothesis cannot be directly addressed at present, given the limitations of current papillomavirus replication systems. However, these findings suggest that papillomavirus may use not only its receptor for attachment and uptake to cells but also the signaling pathway associated with the α6β4 integrin for successful infection. This may represent a general mechanism used by viruses to place cells in a receptive state for viral replication.

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